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14. ABSTRACT

Myelodysplastic syndromes (MDS) are acquired bone marrow failure disorders characterized by poor clinical outcomes due to progressively worsening blood cell counts (especially red blood cells) and increased risk of developing leukemia. MDS originates in hematopoietic stem cells (HSC), but there is little information available regarding the molecular mechanisms regulating MDS HSC function. We have characterized the expression of more than 750 microRNAs in MDS HSC purified from MDS patient samples and compared their expression to normal, age-matched controls. These studies identify at least 31 microRNAs that are differentially expressed in MDS HSC compared to normal controls (FDR >0.1, P<0.05). We have initiated studies to characterize the potential role of these microRNAs in MDS pathogenesis by using a combination of *in vitro* assays and a newly developed xenograft model of MDS. Our findings are consistent with a role of miRNA's in MDS HSC function and future studies will aim to validate the role of each of these candidate miRNAs in MDS development and progression.

15. SUBJECT TERMS
Myelodyplasia, hematopoietic stem cells, microRNA, acute myeloid leukemia, ribosomal protein, mutation, preclinical model, xenotransplantation

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Table of Contents

Introduction: The myelodysplastic syndromes (MDS) are a group of acquired, clonal disorders characterized by ineffective hematopoiesis and increased risk of progression to acute myeloid leukemia (AML). Because HSC are the disease-initiating cells in MDS, identifying the molecular pathways that regulate MDS HSC function will be critical to developing therapies that eliminate the MDS HSC clone. We have performed microRNA expression profiling studies of purified HSC isolated from MDS patient bone marrow samples. Our initial analysis identifies at least 31 differentially expressed microRNA's in MDS HSC when compared with age-matched controls. In addition, we have performed extensive characterization of celluar and functional properties of MDS HSC, providing for the first time insights into the biology of the disease. Our data show that while MDS HSC do not show significant changes compared with their age-matched counterparts with respect to frequency, apoptosis, cell cycle status or engraftment properties in xenograft models, they do exhibit markedly reduced ability to form colonies in vitro. In contrast, committed progenitors in MDSexhibit significant changes compared with their normal counterparts including altered composition, increased apoptosis, and increased cell cycling. Given the presence of miRNA dysregulation and the newly established ability to characterize MDS HSC, we will test the ability of miRNA's to modulate MDS HSC disease phenotypes. Recent studies also suggest that microRNA's (miRNA's) including miR-145 and miR-146a may play primary or cooperating roles in promoting-5q MDS pathogenesis. Potential functional interactions between ribosomopathies and miRNA's have been demonstrated recently in experiments in which knockdown of Rp proteins abrogated miRNA-mediated inhibition of their target genes. Thus, it is possible that in some cases, loss of Rp proteins may affect miRNA function. These findings indicate that miRNA's may play significant roles in MDS HSC biology, and therefore we have proposed a series of experiments that will allow us to not only identify and functionally validate miRNA's important in MDS pathogenesis, but that will also allow us to experimentally model the functional interactons between Rp's and miRNA's. As stated in our original application, we expect our studies to: (1) Identify miRNA's with functional roles in normal and MDS HSC; (2) Identify miRNA's that play roles in MDS progression. These expectations have not changed since our original submission, and we have made significant progress with respect to several of our Aims.

Body/Summary of Findings: Our lab and others have accumulated evidence that the myelodysplastic syndromes (MDS) are a disorder arising in hematopoietic stem cells (HSC), demonstrating the presence of disease specific cytogenetic abnormalities and distinct gene expression programs in purified HSC's ($Lin^-CD34^+CD38^-CD90^+CD45RA^-$) from MDS patients.[1-5] We previously assessed HSC's from five MDS bone marrow (BM) samples by FISH for cytogenetic abnormalities previously identified in each patient. In each of the samples tested, the previously identified chromosomal aberration was present within the HSC population at high frequencies (>84%, data presented in original submission). We have now demonstrated that FACS-purified MDS HSC's can engraft irradiated immunodeficient NOD/SCID/IL2-R null mice (NSG) pup recipients and that the engrafted human cells contain previously characterized cytogenetic lesions. These studies confirm that the HSC is the disease-initiating cell in MDS.

Aberrantly expressed miRNA's have been identified in nearly all hematopoietic malignancies, where they function as oncogenes or tumor suppressors.[7] In MDS, there is evidence that miR-145 and miR-146a contribute to the phenotype of the 5q- syndrome, possibly through inappropriate activation of innate immune signaling.[8,9] Five recent studies have evaluated miRNA expression in non-5q- MDS, identifying dysregulation of a number of miRNA's, including miR-150, miR-181a, miR-221, and miR-222, as well as a ten miRNA signature with prognostic significance.[10-14] Despite these intriguing data, there is no functional data to support a direct pathogenic role for any of these miRNA's in MDS. Furthermore, a, major limitation of these studies to better understanding the role of miRNA's in MDS HSC's was the use of non-purified BM cell populations for analysis. In the past year, two reports have described miRNA expression profiles in $CD34^+$ BM cells from MDS patients,[15,16] finding marked upregulation of the P53 target miR-34a in low risk and 5q- MDS, a new finding that may help explain the increased apoptosis that characterizes MDS. This demonstrates the potential of working with purified cell populations to unmask novel findings. However, even $CD34^+$ selected BM cells represent a highly heterogeneous population composed predominantly of committed hematopoietic progenitors.[17] Because working with heterogeneous cell populations may obscure the true changes that underlie the MDS phenotype at the level of the HSC, we have proposed to evaluated highly purified cells from the $lin^-CD34^+CD38^-CD90^+CD45RA^-$ fraction of bone marrow, which I previously showed to be highly enriched for human HSC's.[18]

We have made significant progress with respect to each of our Specific Aims. These results will be discussed as they were presented in our original SOW.

STATEMENT OF WORK

Task/Aim 1: To determine the functional role of microRNA's in MDS HSC.

1a. Regulatory review and approval process for animal studies. We have successfully obtained regulatory approval for our animal studies. Currently, our 3-yr renewal protocol has been submitted and is pending approval.

1b. To characterize the *in vitro* and *in vivo* functional properties of normal and MDS HSC.
Update: We have developed both *in vitro* and *in vivo* assays to assess MDS HSC function. FACS-sorted MDS HSC do not form significant numbers of colonies (Figure 1). Nevertheless, we have been able to characterize several aspects of MDS HSC biology by flow cytometry:

1) MDS HSC cycle more than normal young HSC, but not more than age-matched controls (Figure 2)
2) MDS HSC do not exhibit increased apoptosis compared to age-matched normal controls, but MDS myeloid progenitors do (Figure 3).
3) MDS bone marrows exhibit significantly reduced frequencies of GMP (Figure 4).

Therefore, we established a xenotransplantation system in which FACS-purified human HSC's are transplanted into newborn immunodeficient NOD/SCID/IL2-R null mice (NSG). We previously reported that using this assay, successful long-term engraftment (>12 weeks) with lympho-myeloid human grafts has been established using as few as 1000 normal HSC's, 1000 HSC's from low-risk MDS, and 2500 HSC's from RAEB-2 MDS (data not shown). We have now gained significant additional experience using this system to characterize basic properties of MDS HSC. When MDS HSC's were transplanted into sublethally irradiated NOD/SCID/IL2Rgamma null (NSG) mice, we were able to establish long-term grafts (Figure 5). In order to demonstrate that the engrafting cells were derived from the transplanted HSC, in cases in which a karyotypic abnormality had been identified, we performed FISH on FACS-sorted cells from engrafted mice. In both cases in which a karyotypic abnormality had been identified, we were able to show that the engrafted HSC harbored the original cytogenetic abnormality (Figure 5). In addition, engrafted committed progenitors exhibited an altered composition with a decrease in the number of immunophenotypically defined GMP (Figure 6). In addition, grafts initiated by MDS HSC were composed almost entirely of myeloid lineage cells with few B-cells produced (Figure 6). While we have not yet achieved engraftment of the originally planned number of samples, we are currently working to expand sample numbers.

1c. To identify differentially expressed miRNA's in MDS HSC compared to normal HSC.
Update: We recently reported a transcriptome analysis of HSC's from eight low-risk MDS patients (refractory anemia, [RA]) with normal cytogenetics and 10 normal controls[20]. This revealed dysregulation of 3,258 mRNAs (FDR <0.1) in MDS, including downregulation of many ribosomal proteins (Figure 7). Among these was RPS6 (40-45% reduction in 8/8 patients), with our subsequent studies demonstrating that shRNA knockdown of RPS6 in human cord blood $CD34^+$ cells leads to a block in erythroid differentiation and disrupts polysomes (Figure 8)[20]. These findings suggest that ribosomal protein insufficiency, previously implicated by Ebert and colleagues in 5q- MDS,[21] may also be a general feature of non-5q- MDS. These compelling findings demonstrate the power of working with purified HSC populations, suggesting that an analysis of miRNA expression in purified HSC's will lead to similarly new insights into MDS biology.

Using the same FACS-purified MDS HSC samples for which we have generated mRNA transcriptome data, we have now begun to generate miRNA expression profiling data using our previously established TaqMan-based qRT-PCR protocol[22]. Comparisons of miRNA expression profiles has been made to age-matched controls, as we have shown that many mRNA transcriptomal changes identified in MDS HSC are not specific to MDS and are instead age-related changes (data not shown). In a preliminary analysis of miRNA expression profiles from HSC's purified from six low-rsk MDS patients (RA) with normal cytogenetics and three normal, age-matched elderly controls, we identified 31 significantly dysregulated miRNA's (FDR <0.1) in MDS HSC's (Figure 9). Among the significantly downregulated miRNA's was miR-125b (mean 171-fold decrease) but not miR-125a. We have previously demonstrated that miR-125b regulates HSC survival through an anti-apoptotic mechanism, leading to a preferential expansion of lymphoid-biased HSC because of their intrinsically higher baseline levels of apoptosis.[23] We propose that downregulation of miR-125b in the MDS HSC contributes to the increased apoptosis that characterizes the MDS BM. Furthermore, preferential apoptosis in lymphoid biased HSC's may explain why the cytogenetic abnormalities, tri-lineage dysplasia, and ineffective hematopoiesis that characterize MDS are not generally found in the lymphoid compartment.[24]

1d. To test the ability of candidate MDS miRNA's to induce MDS-like changes in normal human HSC or rescue MDS HSC.
Update: In preliminary experiments, we have demonstrated that overexpression of an anti-sense "miRZIP" directed towards miR-125b in mouse HSC's *in vitro* leads to increased apoptosis and decreased cellular output, as well as dysplastic maturation of granulocytes (Figure. 10), similar to the phenotype observed in MDS patients. Other miRNA's of interest downregulated in our data set include miR-146a,[8] whose haploinsufficiency appears to contribute to the pathogenesis of the 5q- syndrome by activation of the innate immune system (and interestingly is also downregulated in our cohort of non-5q- patients), and miR-328, which promotes the

translation of the myeloid lineage transcription factor CEBPα.[27] In an analysis comparing common myeloid progenitors from four MDS patients with four normal controls, we did not observe dysregulation of miR-125b, miR-146a, and miR-328, suggesting that the pathogenic significance of these miRNA's is specific to the disease initiating HSC in MDS.

1e: To identify miRNA's associated with MDS disease progression and leukemic transformation.

Milestones, Task 1:

1) Complete miRNA/mRNA expression and functional analysis of HSC from sufficient numbers of MDS samples to generate statistically significant data. **Status: Partially completed**
2) Identify miRNA's and their candidate target mRNAs that are differentially expressed in MDS HSC. **Status: in progress**
3) Identify miRNA's that may mediate the MDS phenotype. **Status: in progress**
4) Identify miRNA's associated with MDS progression and/or transformation. **Status: to do.**

2a. Regulatory review and approval process for animal studies. Animal and IRB protocol approval has been obtained.

2b. To identify common miRNA expression changes in HSC from RPS6 hemizygous mice and MDS HSC.

Update: We are currently in the process of measuring miRNA expression in HSC purified from RPS6 mutant mice; therefore, we have not performed functional experiments proposed in our original proposal. We have, nonetheless, established methods to efficiently introduce lentivirally expressed shRNA's to Rp transcripts into primary cells, demonstrating that human HSC's can be lentivirally transduced with 60-90% efficiency (data not shown). HSC from RPS6 hemizygous mice as well as heterozygous RPS19 missense mutants[28] have been FACS-purified from 3-5 mice and these RNA's are in the process of being prepared for miRNA and mRNA expression profiling studies.

2c: To test candidate MDS miRNA's for enhancement/abrogation of the MDS phenotype in HSC from human or mouse HSC with known ribosomal deficiencies.

Update: Given our initial miRNA expression profiling results identifying miR-125b as a markedly downregulated miRNA in MDS HSC, we will first test whether ectopic miR-125b expression can rescue the MDS HSC phenotype by lentivirally transducing primary MDS HSC with miR-125b. In addition, we have obtained the MDS92 and MDS92L cell lines, which harbor -5q abnormalities in addition to a complex karyotype[29]. While MDS92 cells differentiate spontaneously in the presence of cytokines, thereby mimicking MDS, the MDS92L cell line is maturation arrested and is more similar to transformed AML. Both cell lines offer an alternative system to test the ability of ectopically expressed miRNA's such as miR-125b and miR-145 to rescue aspects of the MDS phenotype, including cell cycling, apoptosis, presence of dysplasia, and decreased erythroid maturation.

2d: To determine whether miRNA induction or reversal of miRNA-like phenotypes in HSC is associated with altered ribosomal protein function.

Update: We have successfully established the ability to generate polyribosome profiles using standard sucrose gradient centrifugation techniques (Figure 8). Using a 10-50% sucrose step gradient, we have been able to generate ribosomal profiles from as few as 50,000 cells (data not shown). Following transduction of miR-125b, miR-145 and empty control lentiviruses into MDS92, MDS92L, or primary MDS cells, we will perform ribosomal profiling as described.

Milestones, Task 2:

1) Determine whether miRNA's and ribosomal proteins function together to contribute to MDS HSC biology. **Status: in progress**
2) Determine the effect of MDS associated miRNA's on ribosomal protein synthesis and ribosome biogenesis. **Status: in progress**
3) Determine if rescue of MDS phenotypes requires restoration of ribosome biogenesis in the ribosomal haplosufficient setting. **Status: to do**

Task 3. Present findings in national meetings and publish in peer-reviewed journals.

3a. Present at national meetings including the American Society of Hematology, Keystone meetings, and International Society for Stem Cell Research Meeting.

3b. Prepare for, and publish, our work for publication in peer-reviewed journals.

Update: We have presented portions of our data at the American Society of Hematology Meeting, Dec 2010, as well as the NIH Myeloid Workshop, Cincinnati, OH, March 2011, and at the FASEB Workshop on Hematologic Malignancies. Our work on the functional and cellular characterization of MDS HSC has been accepted to PNAS and is currently in press.

Milestones, Task 3

1) To present our work in national meetings and publish our findings in scientific journals. **Status: Ongoing**
2) To build future collaborations that will expedite translation of these studies to the diagnostic/therapeutic settings. **Status: To do.**
3) Encourage others to investigate MDS and other hematologic malignancies using purified HSC/committed progenitor subsets. **Status: Ongoing**

Key Research Accomplishments:

- Identification of differentially expressed miRNA's in MDS HSC, which will serve as the basis of functional studies investigating the role of miRNA's in MDS pathogenesis.
- Characterization of MDS HSC frequency, apoptosis, and cell cycle status as well as description of alteration in MDS progenitors that may serve as a future diagnostic test in MDS.
- Establishment of the first xenograft model for purified MDS HSC that allows the characterization of MDS HSC properties, in vivo including lineage bias, engraftment.
- Identification of cell surface markers that are preferentially expressed on MDS HSC, thereby identifying a potential biomarker for diagnosis and tracking of minimal residual disease, as well as a potential novel therapeutic target in MDS.

Reportable Outcomes:

Manuscripts: Paper in press, PNAS (see appendix)
Presentations: NIH Myeloid Workshop, Cincinnati, OH, March 2012.
Training supported by this award: 2012 DOD Bone Marrow Failure Postdoctoral Fellowship awarded to Stephen Chung, MD, fellow in the lab.

Conclusion: We have purified HSC from MDS patients and have characterized them both molecularly and functionally. As originally proposed, we have shown that MDS HSC can be functionally characterized using *in vivo* xenograft models and can recapitulate features of the disease that we have described include a myeloid lineage bias as well as a consistent dropout of GMP. IN addition, we have identified a set of miRNA's that are differentially expressed in MDS HSC versus normal, age-matched controls including miR-125b, a miRNA that we and others have previously shown to regulate HSC self-renewal. Preliminary studies indicate that this miRNA is a potential miRNA that contributes to MDS pathogenesis, as overexpression in human HSC/progenitors results in the generation of mature hematopoietic progeny displaying dysplastic features. Future studies will continue to functionally validate miR-125b and other candidate disease microRNAs using the combination of *in vitro* and *in vivo* assays described in this annual progress report.

References

1. Nilsson L, Astrand-Grundstrom I, Anderson K, et al. Involvement and functional impairment of the CD34(+)CD38(-)Thy-1(+) hematopoietic stem cell pool in myelodysplastic syndromes with trisomy 8. Blood. 2002;100:259-267.
2. Nilsson L, Astrand-Grundstrom I, Arvidsson I, et al. Isolation and characterization of hematopoietic progenitor/stem cells in 5q-deleted myelodysplastic syndromes: evidence for involvement at the hematopoietic stem cell level. Blood. 2000;96:2012-2021.
3. Nilsson L, Eden P, Olsson E, et al. The molecular signature of MDS stem cells supports a stem-cell origin of 5q myelodysplastic syndromes. Blood. 2007;110:3005-3014.
4. Tehranchi R, Woll PS, Anderson K, et al. Persistent malignant stem cells in del(5q) myelodysplasia in remission. N Engl J Med. 2010;363:1025-1037.
5. Will B, Zhou L, Vogler TO, et al. Stem and progenitor cells in myelodysplastic syndromes show aberrant stage specific expansion and harbor genetic and epigenetic alterations. Blood. 2012.

6. Park CY, Pang WW, Price E, Pluvinage JA, Schrier SL, Weissman IL. Hematopoietic Stem Cells Are the Disease-Initiating Cells in the Myelodysplastic Syndromes. ASH Annual Meeting Abstracts. 2011;118:789-.
7. Vasilatou D, Papageorgiou S, Pappa V, Papageorgiou E, Dervenoulas J. The role of microRNAs in normal and malignant hematopoiesis. Eur J Haematol. 2010;84:1-16.
8. Starczynowski DT, Kuchenbauer F, Argiropoulos B, et al. Identification of miR-145 and miR-146a as mediators of the 5q- syndrome phenotype. Nat Med. 2010;16:49-58.
9. Starczynowski DT, Kuchenbauer F, Wegrzyn J, et al. MicroRNA-146a disrupts hematopoietic differentiation and survival. Exp Hematol. 2011;39:167-178 e164.
10. Pons A, Nomdedeu B, Navarro A, et al. Hematopoiesis-related microRNA expression in myelodysplastic syndromes. Leuk Lymphoma. 2009;50:1854-1859.
11. Hussein K, Theophile K, Busche G, et al. Aberrant microRNA expression pattern in myelodysplastic bone marrow cells. Leuk Res. 2010;34:1169-1174.
12. Hussein K, Theophile K, Busche G, et al. Significant inverse correlation of microRNA-150/MYB and microRNA-222/p27 in myelodysplastic syndrome. Leuk Res. 2010;34:328-334.
13. Beck D, Ayers S, Wen J, et al. Integrative analysis of next generation sequencing for small non-coding RNAs and transcriptional regulation in Myelodysplastic Syndromes. BMC medical genomics. 2011;4:19.
14. Sokol L, Caceres G, Volinia S, et al. Identification of a risk dependent microRNA expression signature in myelodysplastic syndromes. British journal of haematology. 2011.
15. Votavova H, Grmanova M, Dostalova Merkerova M, et al. Differential expression of microRNAs in CD34+ cells of 5q- syndrome. Journal of hematology & oncology. 2011;4:1.
16. Dostalova Merkerova M, Krejcik Z, Votavova H, Belickova M, Vasikova A, Cermak J. Distinctive microRNA expression profiles in CD34+ bone marrow cells from patients with myelodysplastic syndrome. European journal of human genetics : EJHG. 2011;19:313-319.
17. Kondo M, Wagers AJ, Manz MG, et al. Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol. 2003;21:759-806.
18. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell. 2007;1:635-645.
19. Park CY, Pang WW, Price E, Pluvinage JA, Schrier SL, Weissman IL. Hematopoietic Stem Cells Are the Disease-Initiating Cells in the Myelodysplastic Syndromes. ASH Annual Meeting Abstracts. 2011;118:789.
20. McGowan KA, Pang WW, Bhardwaj R, et al. Reduced ribosomal protein gene dosage and p53 activation in low-risk myelodysplastic syndrome. Blood. 2011;118:3622-3633.
21. Ebert BL, Pretz J, Bosco J, et al. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. Nature. 2008;451:335-339.
22. Han YC, Park CY, Bhagat G, et al. microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. J Exp Med. 2010.
23. Ooi AG, Sahoo D, Adorno M, Wang Y, Weissman IL, Park CY. MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. Proc Natl Acad Sci U S A. 2010.
24. Nimer SD. MDS: A Stem Cell Disorder—But What Exactly Is Wrong with the Primitive Hematopoietic Cells in This Disease? ASH Education Program Book. 2008;2008:43-51.
25. Vallespi T, Imbert M, Mecucci C, Preudhomme C, Fenaux P. Diagnosis, classification, and cytogenetics of myelodysplastic syndromes. Haematologica. 1998;83:258-275.
26. Bejar R, Stevenson K, Abdel-Wahab O, et al. Clinical effect of point mutations in myelodysplastic syndromes. N Engl J Med. 2011;364:2496-2506.
27. Eiring AM, Harb JG, Neviani P, et al. miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. Cell. 2010;140:652-665.
28. McGowan KA, Pang WW, Bhardwaj R, et al. Reduced ribosomal protein gene dosage and p53 activation in low risk myelodysplastic syndrome. Blood. 2011.
29. Tohyama K, Tsutani H, Ueda T, Nakamura T, Yoshida Y. Establishment and characterization of a novel myeloid cell line from the bone marrow of a patient with the myelodysplastic syndrome. Br J Haematol. 1994;87:235-242.

APPENDICES:

Attached Material:

1) Accepted manuscript on the characterization of MDS HSC (in press at PNAS).
2) Current curriculum vitae.

SUPPORTING DATA: All figures and accompanying legends are attached following the appendix.

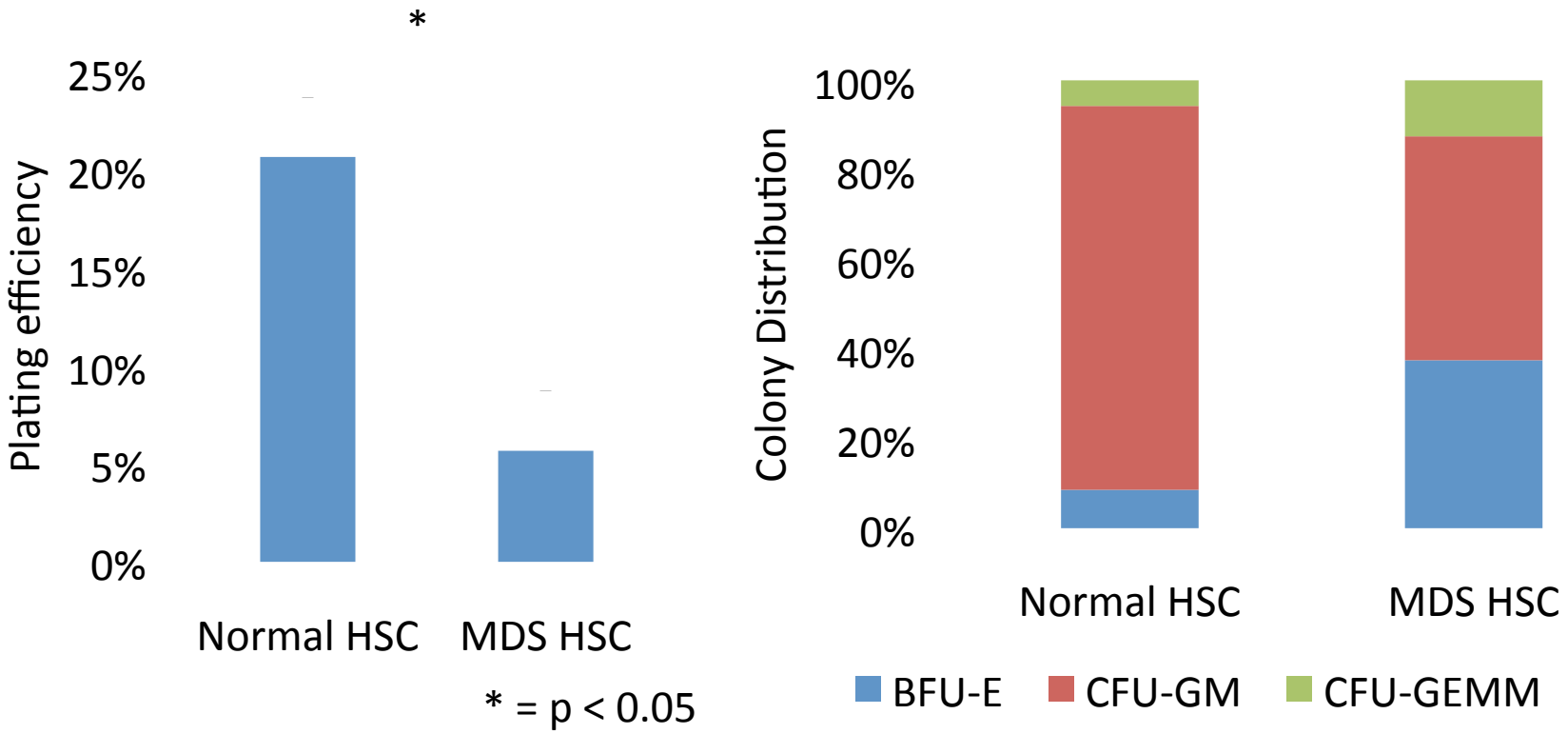


Figure 1. Characteristics of MDS HSC *in vitro*. A) FACS-sorted HSCs were plated into methylcellulose supplemented with complete cytokines (TPO, SCF, FLT3L, IL-6, IL-3, GM-CSF, EPO) and colonies were counted 10-12 days later. Normal HSC, n=10; MDS HSC, n=10. B) Colony subtypes were assessed by morphology.

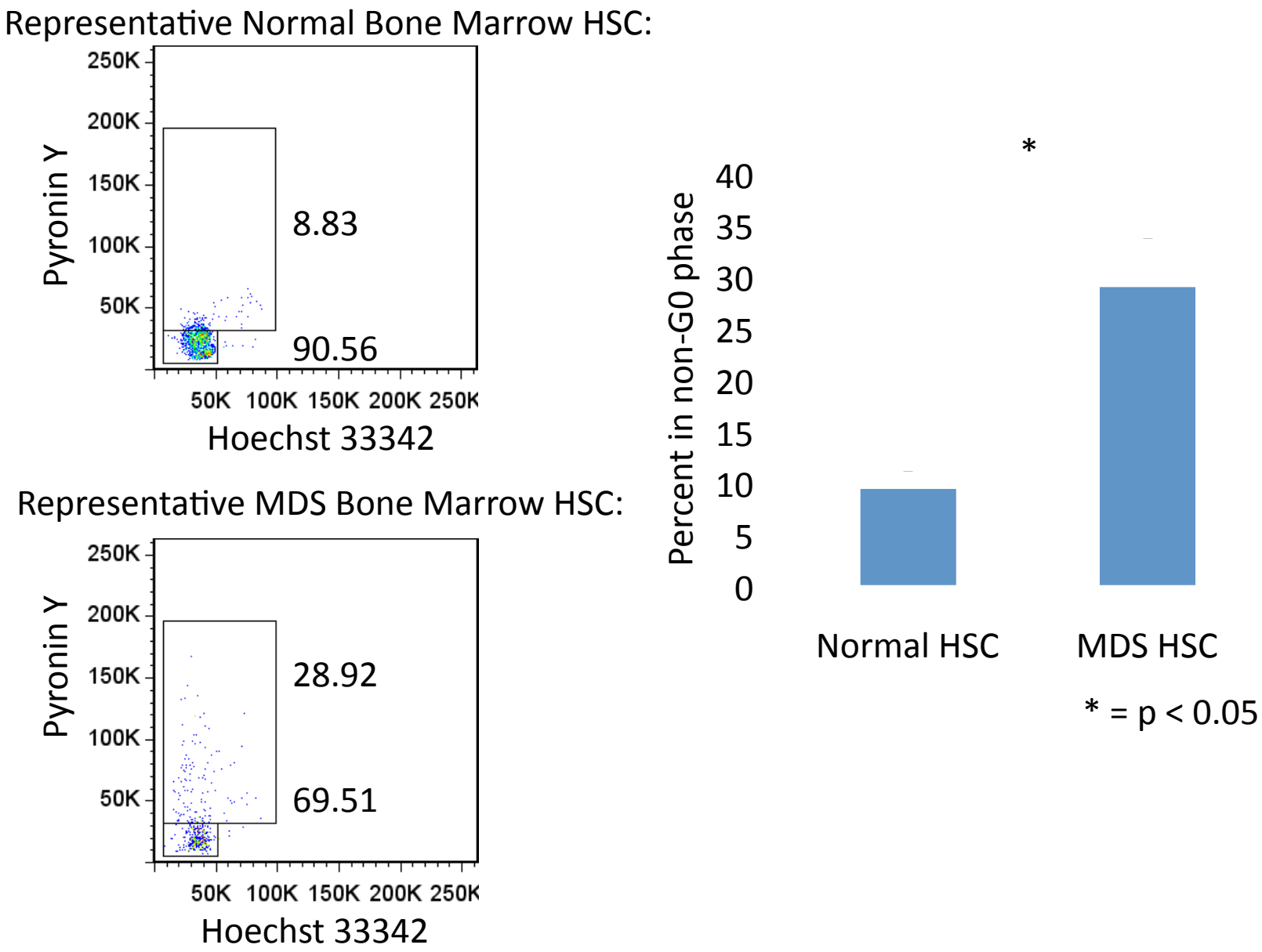


Figure 2. Immunophenotypic HSCs from low grade MDS bone marrow exhibit increased cell cycling. Hoechst 3342 and Pyronin Y staining was performed on MDS bone marrow samples. HSCs were identified based on their immunophenotype (Lin-CD34+CD38-CD90+CD45RA-). Normal HSC, n=12, MDS HSC, n=37.

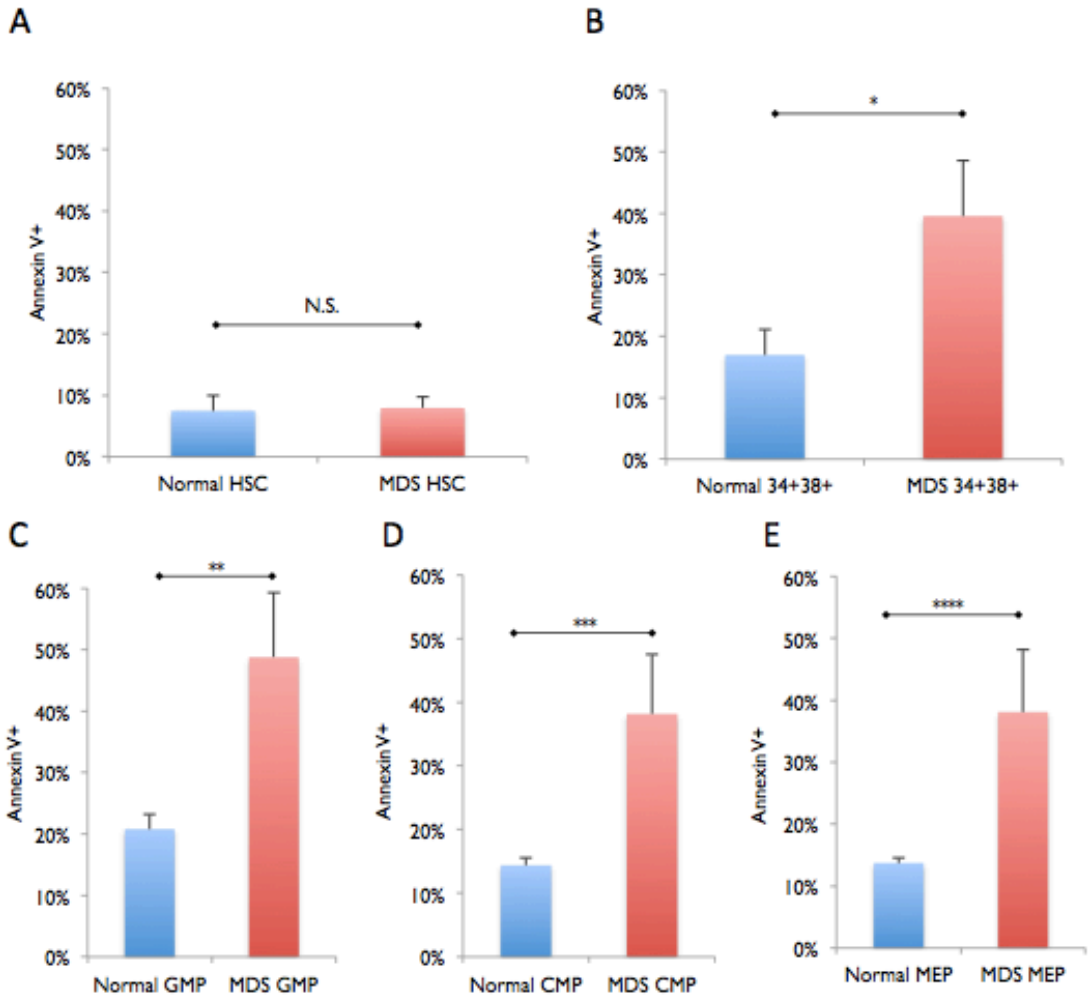
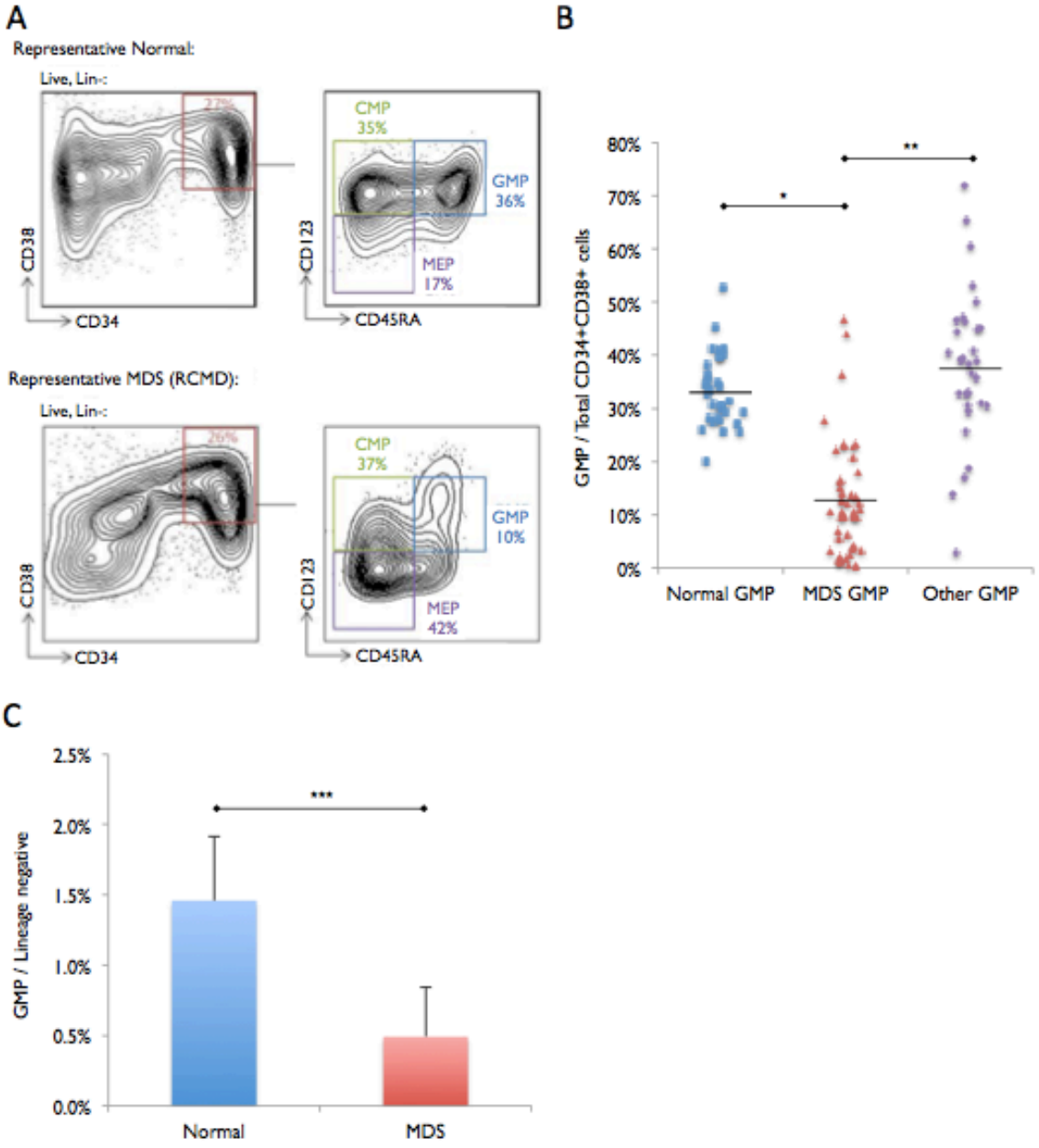


Figure 3. Increased apoptosis in MDS GMPs but not in MDS HSCs

(A) Frequency of Annexin V+ cells in normal and low grade MDS HSCs. (N.S. no significance) (B) Frequency of Annexin V+ cells in normal and low grade MDS CD34+CD38+ myeloid progenitor cells. (*P<0.03) (C) Frequency of Annexin V+ cells in normal and low grade MDS GMPs. (**P<0.02) (D) Frequency of Annexin V+ cells in normal and low grade MDS CMPs. (***P<0.02) (E) Frequency of Annexin V+ cells in normal and low grade MDS MEPs. (****P<0.02) Normal, n=25; MDS, n=37.

Figure 4. Decreased GMP frequency in low grade MDS

(A) Representative gating strategy for normal and MDS CMPs (Lin-CD34+CD38+CD123+CD45RA-), GMPs (Lin-CD34+CD38+CD123+CD45RA+), and MEPs (Lin-CD34+CD38+CD123-CD45RA-). (B) Frequency of GMPs out of total myeloid progenitors (CMPs + GMPs + MEPs) in normal, low grade MDS, and non-MDS with at least one cytopenia bone marrow samples. (*$P<10^{-13}$, **$P<10^{-10}$) (C) Frequency of GMPs out of total lineage negative bone marrow mononuclear cells in normal and low grade MDS bone marrow samples. (***P<0.0006)



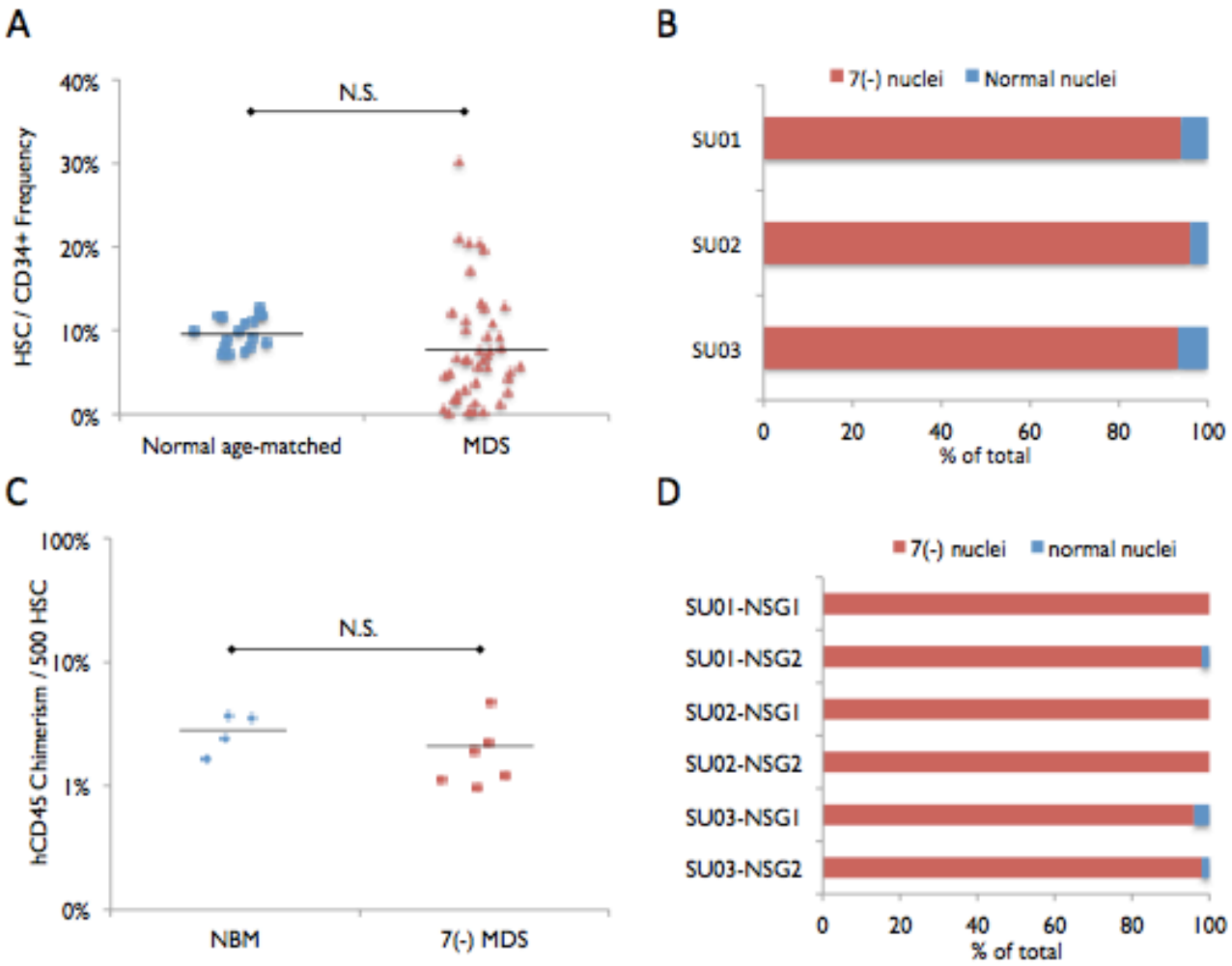


Figure 5. Immunophenotypic HSCs from low grade MDS bone marrow are not increased in frequency and monosomy 7 MDS HSCs reconstitute clonal disease in immunodeficient mice A) Frequency of immunophenotypic HSCs within the CD34+ population in normal age-matched and low grade MDS bone marrow samples. (N.S. no significance) (B) Percentage of HSCs with nuclei exhibiting deletion of chromosome 7 and normal karyotype in monosomy 7 MDS patients (n = 3; SU001 – SU003). 150 nuclei were analyzed for each sample. (C) Human CD45+ chimerism per 500 transplanted HSCs from normal bone marrow controls (n = 4) and monosomy 7 bone marrow samples (n = 3) into sub-lethally irradiated NSG newborn mice (1 recipient per normal HSC sample; 2 recipients per monosomy 7 sample). (N.S. no significance) (D) Percentage of successfully engrafted human CD45+ cells with nuclei exhibiting deletion of chromosome 7 and normal karyotype. HSCs were sorted from 3 monosomy 7 bone marrow samples (SU001 – SU003) and xenotransplanted into 2 recipients each (NSG1, NSG2). 50 human CD45+ nuclei were analyzed from each xenotransplant recipient.

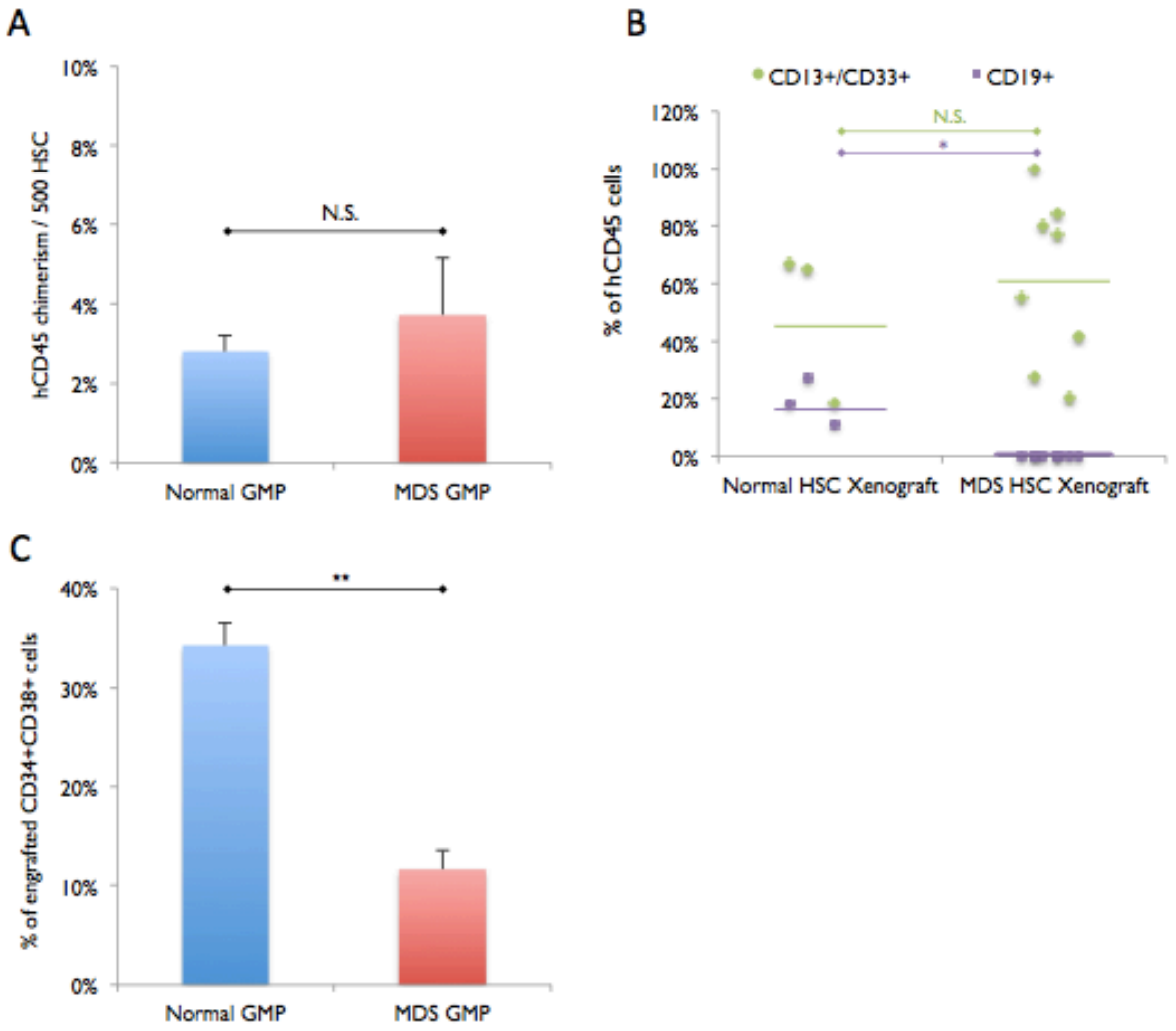


Figure 6. MDS HSCs transplanted into immunodeficient mice yield decreased GMPs and B-cells

(A) Engraftment of 1500-3000 HSCS from normal (n = 4) and low grade MDS (n = 4) bone marrow samples transplanted into NSG newborn mice at 16 weeks post-transplant (1 recipient per normal HSC sample; 2 recipients per low grade MDS sample), as represented by percentage of human CD45+ chimerism per 500 human HSCs transplanted. (N.S. no significance) (B) Lymphoid (CD19+) and myeloid (CD13+/CD33+) engraftment as a percentage of hCD45+ cells in NSG newborn mice at 16 weeks post-transplant. (*P<0.03; N.S. no significance) (C) Frequency of GMPs out of total human CD34+CD38+ cells engrafted in NSG newborn mice at 16 weeks post-transplant. (**P<0.0007)

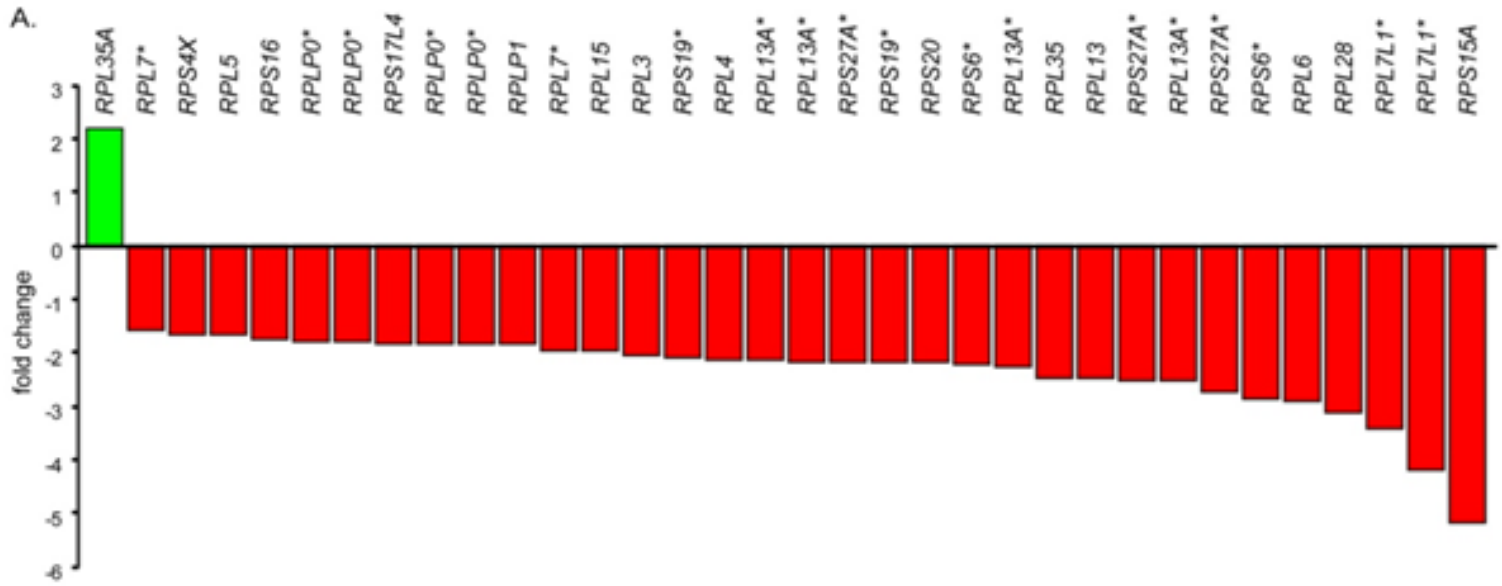


Figure 7. Rp transcripts are frequently downregulated in MDS HSC.
Microarray results for differentially expressed ribosomal protein genes from sorted hematopoietic stem cells from patients with low risk, non-5q- MDS compared to healthy, age-matched controls. Height of bars represents fold change. Most ribosomal protein genes are repressed (red bars) in MDS; green bars indicate induced genes.

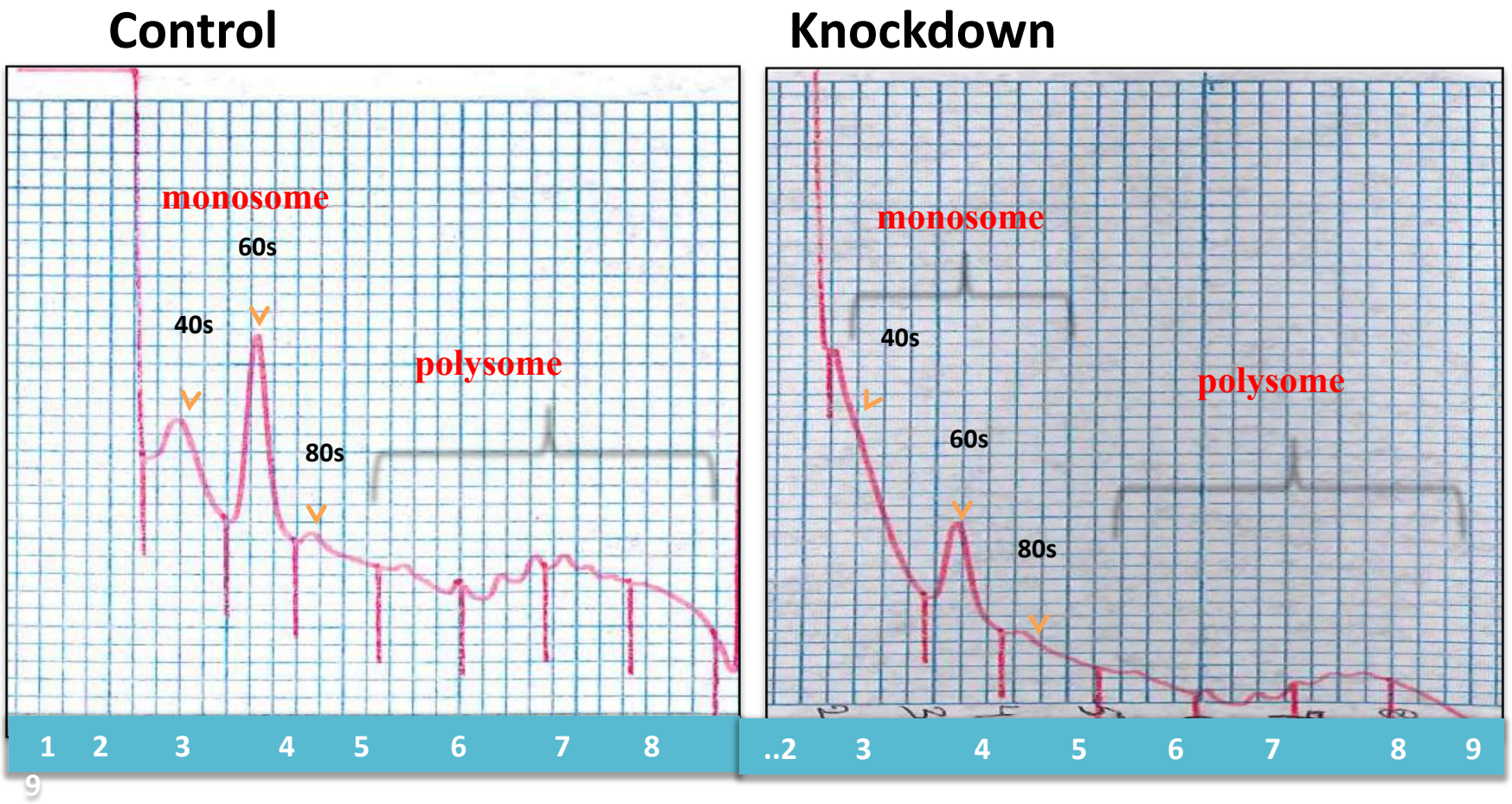
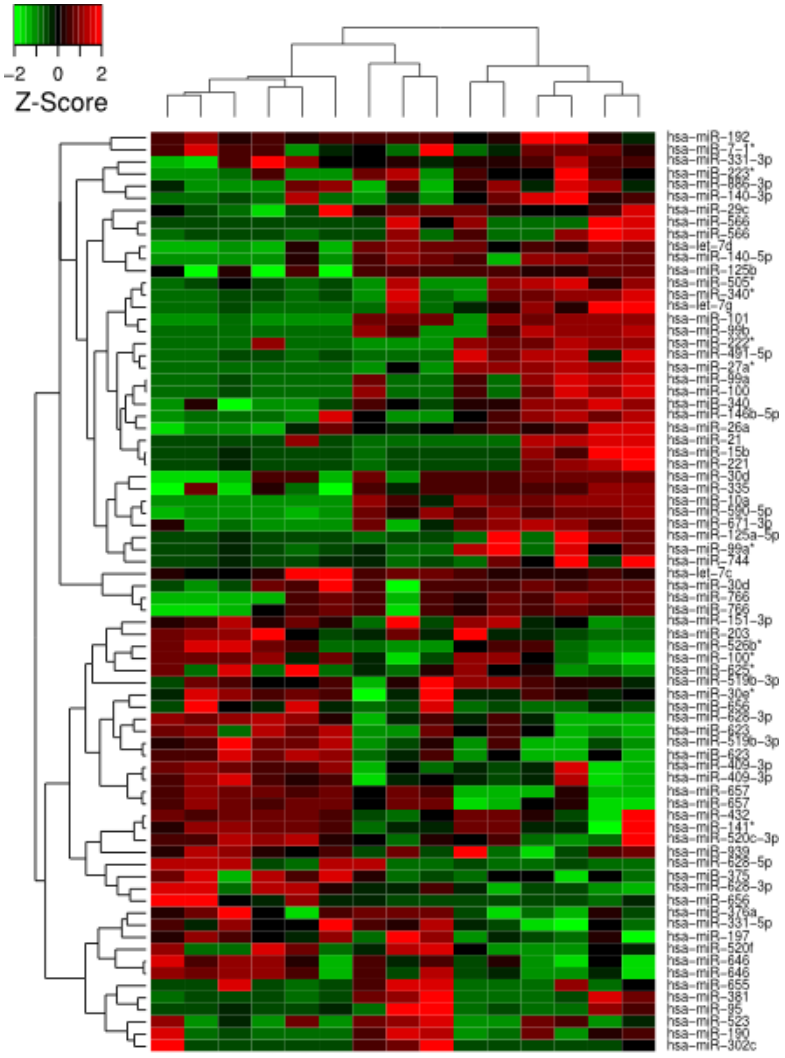
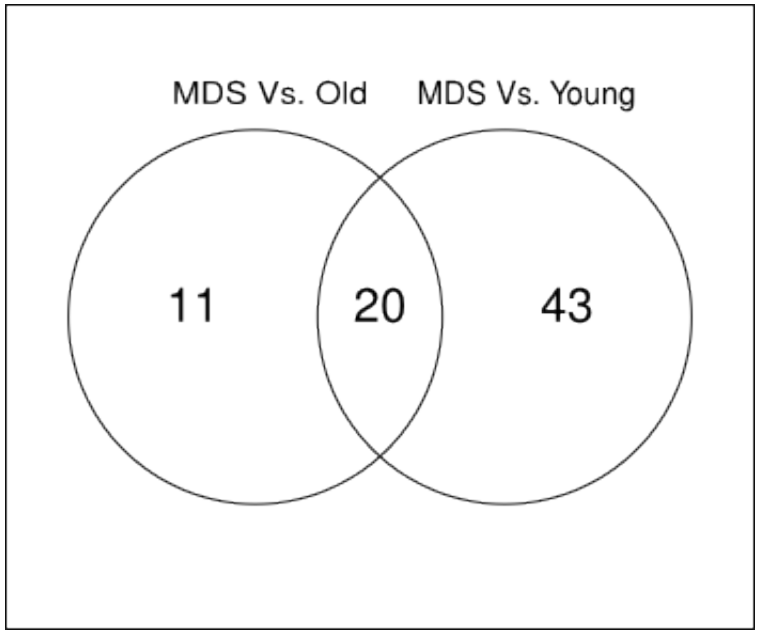


Figure 8. Knockout of RPS6 results in disruption of polysomes. KU812 cells were transduced with a lentivirus expressing an shRNA against Rps6. Three days later, the GFP+ transduced cells were FACS-sorted and polysome profiles were generated using a standard 10-50% sucrose gradient.





P < 0.05

MDS OLD YOUNG

Figure 9. Widespread miRNA Dysregulation in MDS HSC. 76 genes were identified as significant changed from MDS vs. young, or MDS vs old samples based on P Value less than 0.05. Unsupervised analysis demonstrated groups of MDS, old, young samples could be classified by these microRNA signatures, again indicating the important roles of miRs in aging and MDS development. A couple genes that might catch our eyes based on their functional studies, or frequently shown up in microRNA profiling, such as genes that down-regulated in MDS including miR-99a, miR-125b, miR-125a-5p.

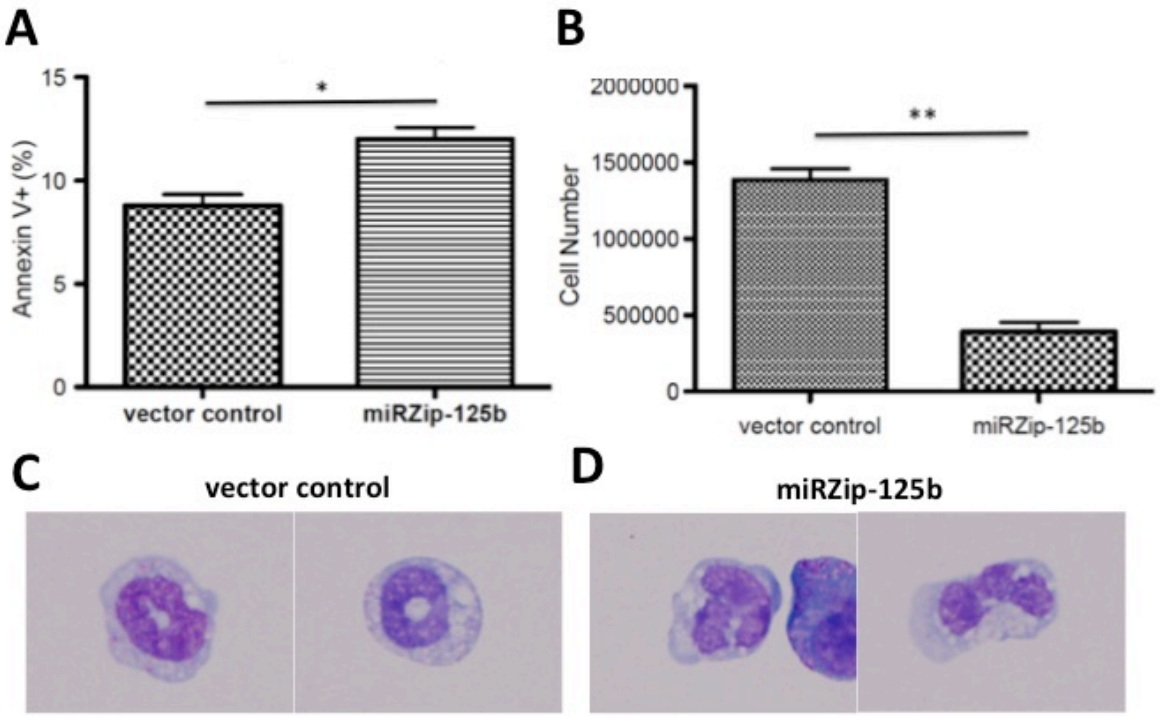


Figure 10. miR-125b knockdown induces dyplastic changes in mouse hematopoietic cells. Mouse hematopoietic stem cells ($lin^-sca\text{-}1^+c\text{-}kit^+CD34^-CD150^+$) were FACS-purified to >95% purity and lentivirally transduced to overexpress an anti-sense miRZip-125b or control vector (transduction efficiency 80% and 85%, respectively) before plating into complete methylcellulose (1000 cells) in triplicate. Cells were harvested after eight days and assessed for apoptosis by Annexin V and 7-AAD staining **(A)** (represented as the percentage of Annexin V positive cells out of total viable 7-AAD negative cells), as well as absolute cell number **(B)** using GFP as a marker of transduction. Wright-Giemsa staining of cytospin preparations of cells transduced with control vector **(C)** or miRZip-125b **(D)** demonstrated the presence of dysplastic hypolobated granulocytes with miRZip-125b expression. *p=0.03, **p=<0.01

On hematopoietic stem cell and progenitor cell mechanisms in myelodysplastic syndrome

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Dedicated to the memory of Bill Walsh, a friend and a famous innovator and mentor at Stanford University

Abstract

Myelodysplastic syndromes (MDS) are a group of disorders likely initiated by hematopoietic stem cells (HSCs), but the variable cytopenias observed in MDS suggests that the loss of downstream progenitors may underlie the ineffective hematopoiesis. HSCs and committed myeloid progenitors in MDS have not been extensively characterized in the laboratory or clinical research settings. We transplanted purified human HSCs from MDS samples with known clonal cytogenetic abnormalities into immunodeficient mice and show that the HSCs engraft and give rise to clonal MDS cells in the mouse bone marrow. We identify a recurrent loss of granulocyte-macrophage progenitors (GMPs) in the bone marrow of low risk MDS patients (fewer than 5% blasts) that can distinguish MDS from its clinical mimics, and can be used prospectively to distinguish low risk MDS, thus providing a novel and simple diagnostic. Transplantation of purified human HSC from low risk MDS samples into immunodeficient mice yields a similar myeloid progenitor profile in engrafted mice, suggesting that HSCs are the disease-initiating cells in MDS. Importantly, the loss of GMPs is likely due to increased apoptosis and increased phagocytosis of this population, the latter due to the up-regulation of cell surface calreticulin (CRT), a pro-phagocytic marker. Blocking CRT on low risk MDS myeloid progenitors rescues this population from phagocytosis *in vitro*. However, in the refractory anemia with excess blasts (RAEB) stage of MDS, the GMP population is increased in frequency compared to normal, and myeloid progenitors evade phagocytosis due to up-regulation of CD47, an anti-phagocytic marker. Blocking CD47 leads to the selective phagocytosis of this population. These results are consistent with the hypothesis that MDS HSC compete with normal HSC in the patients, and that as MDS HSC increase their frequency at the expense of normal hematopoiesis, the loss of MDS myeloid progenitors by programmed cell removal is in part responsible for the cytopenias in this disease; and that up-regulation of the 'don't eat me' signal CD47 on MDS myeloid progenitors is an important transition step leading from low risk MDS to AML. These findings provide evidence for deficient programmed cell removal in the more advance MDS subtypes.

Introduction

The World Health Organization (WHO) defines myelodysplastic syndromes (MDS) as a heterogeneous group of related clonal diseases characterized by variable cytopenias due to ineffective hematopoiesis and increased risk of progression to acute myeloid leukemia (AML)(1). Clonal chromosomal abnormalities are detected in 40-70% of MDS patients(2), and these alterations likely reflect biological differences in MDS pathogenesis, since chromosomal aberrations correlate with patient prognosis(3, 4). With the description and prospective isolation of human hematopoietic stem cells (HSCs) and several classes of progenitors, cell lineage specific alterations in human hematopoietic disorders can now be determined(5-8). Recent FISH and gene expression data from purified HSCs (immunophenotypically defined as Lin-CD34+CD38-CD90+) from 5q- MDS patients have suggested an HSC origin for MDS(9-12). HSCs from MDS patients (MDS HSCs) are relatively resistant to lenalidomide and decitabine treatment, since even patients with cytogenetic remission, as determined by fluorescence *in situ* hybridization (FISH) on peripheral blood cells, can maintain a significant MDS HSC burden(11, 12). Nevertheless, HSCs from MDS patients have not been previously shown to initiate the disease itself.

Additionally, it is not clear whether isolated MDS HSCs exhibit increased apoptosis and aberrant proliferation similar to that observed in total bone marrow cells(13-16). To date, these questions have not been addressed directly, as evaluation of immature hematopoietic cells, in both functional and diagnostic assays, has predominantly relied upon unpurified or partially purified bone marrow cells (most frequently CD34+ cells). These preparations have limited power to evaluate prospectively isolated and functionally validated HSC or committed progenitor populations (reviewed in van de Loosdrecht et al.(17)). We have taken advantage of the purification of highly enriched HSCs and committed myeloid progenitors(7, 8) via flow cytometry and fluorescence activated cell sorting (FACS) to characterize hematopoietic subsets in primary MDS patient bone marrow samples with the goal of increasing the understanding of MDS pathogenesis.

We and others have shown that the binding of cell surface calreticulin (CRT) on a target cell to the low-density lipoprotein-related protein (LRP1) receptor on macrophages is an important mechanism for the phagocytosis of target cells, including apoptotic cells and cancer cells, including AML (18-20). We have also shown that the concomitant up-

regulation of anti-phagocytic CD47 on the surface of cancer cells is critical for their evasion of immunosurveillance(18). In addition, blocking CD47 ligation of its receptor SIRPα allows for the pro-phagocytic cell surface CRT to signal phagocytosis and clearance of tumor cells(18). While cell surface CD47 expression is increased in numerous tumor types, including AML(21-24), these molecules have not been well characterized in MDS.

AML is a multistep disease in which the self-renewing leukemia stem cells (LSCs) emerge at a multilineage progenitor stage. In hematopoiesis, only HSCs self-renew, and therefore we proposed (25-28) and in several cases have shown that the accumulation of genetic and epigenetic changes characteristic of leukemia progression occur in clones of self-renewing HSCs, and not in the downstream progeny that cannot normally self-renew (29-33). It is likely that at least some events in pre-leukemic progression trigger both programmed cell death and programmed cell removal(28). Here we test the notion that low risk MDS (including refractory anemia, refractory anemia with ringed sideroblasts, refractory cytopenia with multilineage dysplasia, and myelodysplastic syndrome not otherwise specified but with fewer than 5% blasts) follow this progression toward high risk MDS (refractory anemia with excess blasts; RAEB, including both WHO RAEB-1 and RAEB-2 subtypes), and reveal a stage wherein the pre-leukemic HSC clones outcompete normal HSC clones, but, at their oligolineage progenitor stage, lose blood-forming capacity as a result of programmed cell death and/or programmed cell removal.

In this study, we demonstrate that low risk MDS HSCs (from bone marrow samples from patients with refractory anemia, refractory anemia with ringed sideroblasts, refractory cytopenia with multilineage dysplasia, and myelodysplastic syndrome not otherwise specified but with fewer than 5% blasts) can transplant the clonal disease into immunodeficient mice. We also show that fewer granulocyte-macrophage progenitors (GMPs) are present in low risk MDS bone marrow compared to normal and non-MDS bone marrow samples, a quantitative finding that can be used to aid diagnosis of MDS in the clinical setting. This reduction in GMPs is likely secondary to increased programmed cell death and programmed cell removal by macrophages, the latter of which is due to up-regulation of CRT. In high risk refractory anemia with excess blasts (RAEB) stage of MDS,

the frequency of GMPs in the bone marrow is higher than in low risk MDS, likely due to their evasion of phagocytosis by up-regulation of CD47.

Results

Similar frequency of HSCs in low risk MDS bone marrow and normal bone marrow

MDS is thought to be a disease of HSCs, leading to variable cytopenias due to ineffective production of mature granulocyte, erythroid, and/or megakaryocyte (platelet) populations(1). However, there is little information regarding the status of the most primitive hematopoietic stem and progenitor compartments in MDS bone marrow. We used flow cytometry based on immunophenotypic markers to evaluate the distribution and frequency of HSCs (Lin-CD34+CD38-CD90+CD45RA-)(5, 34-38), multipotent progenitors (MPP; Lin-CD34+CD38-CD90-CD45RA-)(8) and committed myeloid progenitor populations(7) in low risk MDS and non-MDS bone marrow. The use of age-matched controls is crucial in studies such as this, since most patients with MDS are elderly, and normal bone marrow changes with age. Specifically we have shown that the frequency of immunophenotypic HSCs in normal bone marrow is significantly higher in the elderly (ages 65 years and older) than in the young (ages 20-35 years)(39). Therefore we compared the frequencies of immunophenotypic HSCs in low risk MDS patients and similarly aged (elderly), non-anemic normal controls. We found that the frequencies of these cells were not significantly higher in low risk MDS bone marrow than in normal bone marrow (Figure 1a). These findings suggest that MDS HSCs expand at the expense of normal HSCs.

HSCs from MDS bone marrow can transplant clonal disease into immunodeficient mice

Previously, it has been shown that the HSC compartment in MDS is enriched for cytogenetically abnormal cells, including in two MDS samples with monosomy 7(9, 10, 12). We have evaluated three MDS samples harboring a clonal monosomy 7 cytogenetic abnormality. Consistent with previous reports, we can detect this cytogenetic abnormality by FISH in greater than 95% of the cells within the immunophenotypically defined HSC population (Figure 1b). We then purified the HSCs by FACS and xeno-transplanted them into sublethally irradiated immunodeficient NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) pups(40). NSG mice transplanted with MDS HSC were sacrificed at 12-16 weeks and human chimerism was measured by the presence of human CD45+ cells in the bone marrow of all the recipient mice and expressed as human chimerism per 500 HSCs transplanted (Figure

1c). Human CD45+ cells were then FACS-purified from these engrafted mice and analyzed by FISH. We find that the human CD45+ cells from these engrafted mice harbor the cytogenetic abnormality (Figure 1d), showing that HSCs from MDS initiate and carry the abnormality and can transplant and initiate this clonal disease. Interestingly, the frequency of cells derived from the abnormal MDS clone was similar to that observed in the original patient bone marrow sample, indicating that the MDS clone did not exhibit reduced reconstitution ability in the context of transplant.

Decreased granulocyte-macrophage progenitor frequency in low risk MDS compared to normal elderly bone marrow

Because the frequency of HSCs in MDS patient bone marrow samples is not significantly different compared to normal age-matched controls and simple loss of HSCs cannot directly explain the several cytopenias that are found in this disease, we hypothesized that the loss of downstream myeloid progenitors plays a role in the ineffective hematopoiesis characteristic of MDS. In order to characterize changes in the myeloid progenitor populations in MDS, we evaluated by flow cytometry the distribution and frequency of immunophenotypic common myeloid progenitors (CMPs; Lin-CD34+CD38+CD123+CD45RA-), granulocyte-macrophage progenitors (GMPs; Lin-CD34+CD38+CD123+CD45RA+), and megakaryocyte-erythroid progenitors (MEPs; Lin-CD34+CD38+CD123-CD45RA-)(7) in low risk MDS and normal bone marrow controls (Figure 2a; Supplemental Figure 2). A recent study has shown a relative increase in CMP frequency in MDS(12). We found that low risk MDS bone marrow exhibits alterations in myeloid progenitor distribution, with significant reduction of GMP frequency in low risk MDS compared to normal controls ($p<10^{-13}$) as well as non-MDS patients exhibiting at least one cytopenia ($p<10^{-10}$) (Figure 2a, b). When the frequency of GMPs as a percentage total of myeloid progenitors (GMP/[CMP+GMP+MEP]) was calculated, it was decreased by an average of 2.6-fold (Figure 2b) and was accompanied by concomitant increases in relative CMP frequency compared to non-MDS controls (Supplemental Figure 1a). Interestingly, the loss of GMPs in low risk MDS bone marrow was not regularly accompanied by absolute neutropenia in the peripheral blood. Notably, the most common cytopenia in MDS is anemia, but we found that MDS MEPs are not significantly reduced in frequency relative to

the total myeloid progenitor population (Supplemental Figure 1b). We do not yet have markers that define pure megakaryocytic or pure erythroid committed progenitors, and although we suspect these will be altered in the marrow of MDS patients with respective thrombocytopenias and anemias, we cannot test that hypothesis directly at this time.

Accurate absolute quantification of HSC and progenitor numbers from human bone marrow samples is challenging due to the inherent variability in bone marrow aspiration technique, which always collects a varying proportion of cells from the peripheral blood. Therefore, to better approximate absolute numbers of myeloid progenitor subsets in the bone marrow, we compared frequencies of CMPs, GMPs, and MEPs within the lineage-negative population, which are more likely to reflect hematopoietic cells from the bone marrow than total cells collected from bone marrow aspiration. We found that low risk MDS GMPs are decreased in frequency, on average by 3.0-fold, within the lineage-negative population compared to normal ($p<0.0006$) (Figure 2c), whereas low risk MDS CMPs and MEPs are not increased in frequency within the lineage-negative population (Supplemental Figure 1c, d), suggesting that one of the defects in MDS hematopoiesis is not due to an absolute expansion of the CMP or MEP populations, but a significant absolute reduction in the GMP population.

GMP-to-myeloid progenitor ratios predictive of low risk MDS

Because we observed a consistent, statistically significant decrease in the frequency of GMPs among total myeloid progenitors, we tested whether or not GMP frequency could be used as a clinically useful tool to diagnose low risk MDS. Using the gating strategy depicted in Figure 2a, we determined the frequencies of GMPs, CMPs, and MEPs for MDS patients and normal controls. Setting a threshold of 24.0% frequency of GMPs among the myeloid progenitors yielded a sensitivity of 92% and a specificity of 89% for correctly identifying low risk MDS patients. The reduced GMP frequency observed in low risk MDS was rarely seen in bone marrow samples from non-MDS bone marrow disorders. We evaluated, after determination of our initial threshold of 24.0% GMPs for low risk MDS diagnosis, the myeloid progenitor profile of 40 consecutive bone marrow samples submitted for evaluation of MDS and correctly categorized 100.0% of 28 non-MDS samples

(false positive rate of 0.0) and 83.3% of 12 low risk MDS samples in a blinded fashion (false negative rate of 0.067).

While many diagnostic flow cytometry labs are beginning to employ 6-color flow cytometry into their clinical practice, many laboratories still do not possess the ability to evaluate more than 4 colors simultaneously. Because identification of myeloid progenitors typically requires at least 5 colors (Lin, CD34, CD38, CD123, CD45RA), we re-evaluated the same flow cytometry data files using only 4 colors to identify "pseudo-" or "surrogate" GMP (CD34+, CD38+, CD123+, CD45RA+), CMP, and MEP populations. Unfortunately, using this strategy, attempting to gate on myeloid progenitors without performing a pre-gate on Lin- cells did not result in statistically significant differences in low risk MDS and non-MDS samples, possibly due to the variable inclusion of peripheral blood cells retrieved during bone marrow aspiration.

Decreased frequency of GMPs is an intrinsic characteristic encoded within low risk MDS HSCs

To further determine whether the decreased frequency of GMPs is an inherent feature of low risk MDS, we transplanted FACS-purified HSCs from low risk MDS bone marrow samples into sublethally irradiated NSG pups, and analyzed the distribution of myeloid progenitors in the bone marrow of engrafted mice. As controls, we transplanted FACS-purified HSCs from normal human bone marrow samples. Human CD45+ chimerism was detected at 12-16 weeks in mice transplanted with MDS HSCs as well as normal HSCs (Figure 3a). In mice transplanted with low risk MDS HSCs, engrafted cells were predominantly derived from the myeloid lineage, as evidenced by CD13+ and/or CD33+ staining, and minimally in B-lymphoid lymphoid lineage, as evidenced by CD19+ staining, whereas mice transplanted with normal HSCs exhibited comparatively more robust engraftment in the B-lymphoid lineage (Figure 3b). Human CD34+ cells were also detected in the bone marrow of engrafted mice, and GMPs as a frequency of human CD34+ cells were decreased in mice transplanted with low risk MDS HSCs compared to mice transplanted with normal HSCs (Figure 3c). These data indicate that the low risk MDS HSCs can transfer a human graft that recapitulates features present in MDS patient bone marrow

samples. Furthermore, it suggests that the relative loss of GMP is due to a cell-intrinsic defect in MDS HSCs.

Apoptosis of low risk MDS progenitor cells but not hematopoietic stem cells

Numerous prior studies characterizing CD34+ cells, of which only a small fraction are putative HSCs, in the bone marrow of MDS patients revealed two hallmarks of MDS—increased apoptosis and a concomitant increased in the proliferative fraction; however, these data provide no specific information regarding whether or not HSCs or specific progenitor populations are pathologically affected in MDS. In order to determine whether these changes affect all immature hematopoietic cell populations, we evaluated apoptosis in low risk MDS patient samples and controls by flow cytometry by measuring annexin V binding. Normal and low risk MDS HSCs showed similar levels of annexin V staining (Figure 4a), but Lin-CD34+CD38+ cells, comprised of myeloid and lymphoid progenitors, showed a statistically significant 2.3-fold increase in the frequency of annexin V-positive cells ($p < 0.03$) (Figure 4b). Additionally, in order to determine whether all myeloid progenitor subsets in low risk MDS patients exhibited increased apoptosis, we measured annexin V staining of CMPs, GMPs, and MEPs from normal and MDS bone marrow samples. CMPs, GMPs, and MEPs from low risk MDS all exhibited a statistically significant increase in the frequency of annexin V-positive cells ($p<0.02$) (Figure 4c, d, e). These data indicate that while MDS is a disease originating in HSC, the increased apoptosis observed in MDS does not manifest in HSCs but rather in downstream myeloid progenitors.

Increased calreticulin expression in low risk MDS progenitor cells

In addition to increased apoptosis, another mechanism that may contribute to the reduced frequency of GMPs observed in low risk MDS patients is increased phagocytosis. Therefore, we measured the cell surface expression of the pro-phagocytic marker CRT in normal and low risk MDS bone marrow. When we examine the HSC population, we find that cell surface expression of CRT is similar between normal and low risk MDS HSCs (Figure 5a). In contrast, we found that cell surface expression of CRT is on average 2.3-fold higher on low risk MDS bone marrow CD34+CD38+ oligolineage progenitor cells compared to normal ($p<0.02$) (Figure 5b). Furthermore, low risk MDS GMPs exhibit significantly

higher levels of pro-phagocytic cell surface CRT expression compared to normal GMPs ($p<0.05$) (Figure 5c). These findings suggest that increased CRT expression on progenitor cells, and GMPs in particular, is a definitive feature of low risk MDS.

Interestingly, while low risk MDS CMPs also demonstrate an increased, albeit not statistically significant, cell surface CRT expression (Supplemental Figure 2a), their progeny, low risk MDS MEPs, do not show this increase in cell surface CRT expression (Supplemental Figure 2b). It is not clear whether the low risk MDS MEPs are CRT-low because CRT is not translocated to the surface of these cells, or if the CRT-high MEPs have already been removed by phagocytosis.

Low risk MDS CD34+CD38+ progenitor cells undergo CRT-mediated phagocytosis by human macrophages more efficiently than normal CD34+CD38+ progenitors

Because specific progenitor populations within low risk MDS bone marrow cells exhibit increased cell surface CRT expression, we explored the hypothesis that these cells would be more susceptible to CRT-mediated phagocytosis by macrophages. Thus, we performed *in vitro* phagocytosis assays on CD34+CD38+ bone marrow cells from normal and low risk MDS samples. Upon incubation with normal human macrophages, CD34+CD38+ cells from low risk MDS bone marrow were on average 1.8-fold more robustly phagocytosed than CD34+CD38+ cells from normal bone marrow ($p<0.02$) (Figure 5d). Interestingly, CD34- cells from low risk MDS bone marrow samples were not as readily phagocytosed by human macrophages *in vitro* compared to both CD34+CD38+ cells from low risk MDS bone marrow as well as CD34+CD38+ and CD34- cells from normal bone marrow (Supplemental Figure 3).

To further explore the role of CRT-mediated phagocytosis in low risk MDS, we determined whether inhibition of cell surface CRT prevents phagocytosis of low risk MDS CD34+CD38+ progenitor cells by human macrophages. While phagocytosis of CD34+CD38+ cells from low risk MDS bone marrow samples was robust at baseline, incubation of low risk MDS and normal CD34+CD38+ cells with a CRT-blocking peptide, which acts by inhibiting the CRT-LRP interaction that signals phagocytosis, resulted in significant abrogation of phagocytosis by an average of 2.6-fold ($p<0.003$) (Figure 5e). These findings support the idea that increased cell surface CRT expression on CD34+CD38+ progenitor

cells results in their increased CRT-mediated programmed cell removal in low risk MDS bone marrow.

CD34+CD38+ progenitor cells from RAEB MDS express increased CD47 compared to low risk MDS CD34+CD38+ progenitor cells

Given that CD47 is an anti-phagocytic marker that counterbalances the pro-phagocytic signal from cell surface CRT, we investigated the expression of CD47 on CD34+CD38+ progenitors from low risk MDS as well as high risk MDS (RAEB). Of note, the immunophenotypic GMP population is not reduced in RAEB, and this population is predominantly composed of blasts (Supplemental Figure 4a). We first measured the cell surface expression of CD47 on low risk MDS CD34+CD38+ progenitors and found that CD47 expression is not significantly increased compared to normal CD34+CD38+ progenitors (Supplemental Figure 4b). We also investigated CD47 expression on low risk MDS CMPs, GMPs, and MEPs, and found that CD47 expression in these populations is similar in low risk MDS compared to their normal counterparts (Supplemental Figure 4c). However, when bone marrow samples from patients with high risk MDS (RAEB) were evaluated, CD34+CD38+ progenitors were found to express an average 3.6-fold increase in CD47 compared to this same immunophenotypic population in low risk MDS and normal bone marrow samples ($p<0.03$) (Figure 6a). Moreover, the blasts within the CD34+CD38+ population from RAEB exhibit significantly increased expression of CD47 ($p<0.03$) (Figure 6b). Of note, CD34+CD38+ cells from RAEB also express increased cell surface CRT compared to normal CD34+CD38+ cells ($p<0.04$) (Figure 6c), and it is the blast population that has significantly higher expression of cell surface CRT ($p<0.04$) (Figure 6d).

These observations lead us to hypothesize that CD34+CD38+ cells from RAEB are able to evade phagocytosis by human macrophages. Indeed, incubation with human macrophages showed that CD34+CD38+ cells from RAEB are not phagocytosed with the same efficiency as CD34+CD38+ cells from low risk MDS ($p<0.03$) (Figure 6e). Importantly, addition of a blocking anti-CD47 antibody (B6H12) resulted in significantly increased phagocytosis of CD34+CD38+ cells from RAEB ($p<0.006$) (Figure 6e). In contrast, addition of CRT-blocking peptide does not alter the ability of macrophages to phagocytose CD34+CD38+ cells from RAEB (Figure 6e). These findings suggest that transformation of

low risk MDS to high risk MDS (RAEB) likely involves the up-regulation of anti-phagocytic CD47 signal to counterbalance the expression of pro-phagocytic cell surface CRT. It is conceivable that this is the ultimate event in the progression of MDS to RAEB and possibly AML, and therefore that the increased expression of CD47 on MDS blasts heralds emergence of the fully malignant clone.

Discussion

We have examined the composition of HSC and myeloid progenitor populations from bone marrow samples of MDS patients as well as age-matched normal and non-MDS cytopenic controls. The HSC population in MDS patient bone marrow is not increased in frequency, on average, when compared to age-matched normal control HSC populations. Additionally, MDS bone marrow samples harboring a clonal cytogenetic marker, such as monosomy 7, contain immunophenotypic HSCs that are composed almost entirely of the MDS clone, but a small fraction of the HSC population are not part of the MDS clone and therefore may represent normal or even pre-MDS clones. These data suggest that MDS HSCs are expanding at the expense of normal HSC, much as we have shown for immunophenotypic HSCs in the chronic phase of CML(29). However, the frequency of HSCs in MDS bone marrow shows greater variance than HSCs from normal age-matched bone marrow, which may reflect how well the MDS HSC population from a given patient is expanding into existing or possibly new HSC niches due to differences in the genetic subtypes of MDS. The sample size was not sufficient to identify significant clinical correlations with MDS HSC frequency.

We have shown that purified immunophenotypic MDS HSCs can initiate clonal MDS in the bone marrow and peripheral blood cells of transplanted immunodeficient mice. We have previously demonstrated that normal elderly (age 65 years and older) HSCs in the same xenotransplantation model are inherently biased towards myeloid differentiation and exhibit limited lymphoid differentiation potential compared to normal young (age 20-35 years) HSCs(39). Since MDS prevalence increases with age, it is possible that the bias of normal elderly HSCs away from lymphoid differentiation predisposes them to develop into MDS HSCs, which we have shown to exhibit even poorer generation of lymphoid progeny, a phenotype that has been described in human MDS patients(41). MDS HSC xenografts also generate reduced frequencies of GMPs, consistent with the hypothesis that MDS HSCs are not only the disease initiating cell in MDS but also suggests that lineage bias and the phenotype of ineffective hematopoiesis are largely cell-intrinsic. We cannot completely exclude the possibility that the mouse recipient microenvironment may contribute to these phenotypes.

Our evaluation of MDS HSCs and progenitors reveals that a significant decrease in the frequency of GMPs is a common characteristic of MDS bone marrow, both in our initial analysis as well as in our prospective, blind evaluation of 40 patient samples submitted for evaluation of MDS to a clinical flow cytometry service. Notably, in immunodeficient mice transplanted with MDS HSC, we also observed a similar reduction in GMPs in the human myeloid progenitor profile reflected in the bone marrow of engrafted mice. As the MDS samples tested were randomly selected, this indicates that these findings are likely true in multiple genetic subtypes of MDS and that different subtypes of MDS share certain common pathophysiologic characteristics; however, additional prospective studies using genetically characterized MDS samples will be required to verify this hypothesis.

We were particularly interested in determining whether MDS HSCs exhibit differences compared to normal HSCs, since MDS HSC are expected to exhibit functional alterations that promote their expansion in the bone marrow. As most prior evaluations of immature hematopoietic cells have relied on evaluation of CD34+ cells, specific changes in MDS HSCs have remained elusive. We have determined that MDS myeloid progenitors, and not MDS HSCs, exhibit increased apoptosis, as illustrated by increased annexin V staining. In addition, CD34+CD38+ hematopoietic progenitor cells, including GMPs, from MDS patients express increased cell-surface CRT, a pro-phagocytic receptor, and inhibition of cell-surface CRT reduces their phagocytosis by macrophages. While CRT has been implicated as a pro-phagocytic signal in numerous tumor types(18), its expression has not been well characterized in MDS. As CRT is not up-regulated at the most primitive level of the HSC but only downstream beginning at the more committed progenitor stages, these results indicate that loss of progeny from maturing progenitors such as GMPs is likely the major source of cell loss in MDS. By blocking the CRT-LRP interaction using a CRT-blocking peptide, we have shown that phagocytosis of MDS progenitor cells can be abrogated. The increased phagocytosis and apoptosis of GMPs may explain the reduced frequency of GMPs in MDS bone marrow and possibly also the neutropenia observed in the peripheral blood of 15-20% of MDS patients. The other cytopenias in MDS, anemias and thrombocytopenias, are not explained by a significant loss of MEPs. However, compared to normal MEPs, MDS MEPs exhibit increased apoptosis, as evidenced by an increased frequency of annexin V-positive cells, which may account in part for the anemias and thrombocytopenias

commonly observed in MDS patients. We predict that downstream pure erythroid and/or megakaryocytic progenitors exhibit increased programmed cell death and/or programmed cell removal in MDS. Notably, because MDS HSCs do not up-regulate CRT, they are not likely to be eliminated by phagocytosis and therefore persist and expand in the bone marrow, perpetuating disease.

The importance of macrophage-mediated phagocytosis in MDS pathogenesis is further supported by our finding that that CD34+CD38+ hematopoietic progenitor cells from high risk MDS (RAEB) bone marrow samples express increased CD47, a cell surface marker we and others have shown is a critical anti-phagocytic marker that counteracts the pro-phagocytic cell surface CRT signal(18-24). In this study, we have shown that low risk MDS progenitor cells express increased levels of cell surface CRT but not CD47, compared to normal controls, and are therefore susceptible to phagocytosis and clearance by macrophages. In contrast, MDS variants that have progressed to RAEB express high levels of CD47, and can avoid phagocytosis, likely promoting their ability to propagate, and gradually take over the bone marrow. We propose that up-regulation of CD47 is an important step in the transformation of low risk MDS into RAEB and possibly AML.

In summary, we propose that the events that characterize the early stages of MDS result from MDS HSCs expanding in competition with normal HSC, yet losing oligolineage progenitors by programmed cell death and/or programmed cell removal and resulting in peripheral cytopenias. These data provide an explanation as to why do so many different mutations, translocations, and likely epigenetic alterations can give rise to MDS; these genetic and epigenetic insults ultimately up-regulate programmed cell death and programmed cell removal(28, 42). We propose that as the MDS clones lose programmed cell death, the cytopenias still result from programmed cell removal. Finally, in the late stages of progression resulting in RAEB and AML, there is the loss of programmed cell removal by expression of the “don’t eat me signal,” CD47.

Figure Legends

Figure 1. Immunophenotypic HSCs from low risk MDS bone marrow are not increased in frequency and monosomy 7 MDS HSCs reconstitute clonal disease in immunodeficient mice

(A) Frequency of immunophenotypic HSCs within the CD34+ population in normal age-matched (n = 18) and low risk MDS (n = 45) bone marrow samples. (N.S. no significance) (B) Percentage of HSCs with nuclei exhibiting deletion of chromosome 7 and normal karyotype in monosomy 7 MDS patients (n = 3; SU001 – SU003). 150 nuclei were analyzed for each sample. (C) Human CD45+ chimerism per 500 transplanted HSCs from normal bone marrow controls (n = 4) and monosomy 7 bone marrow samples (n = 3) into sub-lethally irradiated NSG newborn mice (1 recipient per normal HSC sample; 2 recipients per monosomy 7 sample; 1500-3000 HSCs transplanted per recipient). (N.S. no significance) (D) Percentage of successfully engrafted human CD45+ cells with nuclei exhibiting deletion of chromosome 7 and normal karyotype. HSCs were sorted from 3 monosomy 7 bone marrow samples (SU001 – SU003) and xenotransplanted into 2 recipients each (NSG1, NSG2). 50 human CD45+ nuclei were analyzed from each xenotransplant recipient.

Figure 2. Decreased GMP frequency in low risk MDS

(A) Representative gating strategy for normal and MDS CMPs (Lin-CD34+CD38+CD123+CD45RA-), GMPs (Lin-CD34+CD38+CD123+CD45RA+), and MEPs (Lin-CD34+CD38+CD123-CD45RA-). (B) Frequency of GMPs out of total myeloid progenitors (CMPs + GMPs + MEPs) in normal (n = 34), low risk MDS (n = 46; MDS), and non-MDS with at least one cytopenia bone marrow samples (n = 32; Other). (*$P<10^{-13}$, **$P<10^{-10}$) (C) Frequency of GMPs out of total lineage negative bone marrow mononuclear cells in normal and low risk MDS bone marrow samples. (***$P<0.0006$) Error bars represent one standard deviation. “MDS” label represents low risk MDS samples.

Figure 3. Low risk MDS HSCs transplanted into immunodeficient mice yield decreased GMPs

(A) Engraftment of 1500-3000 HSCS from normal (n = 4) and low risk MDS (n = 4) bone marrow samples transplanted into NSG newborn mice at 16 weeks post-transplant (1 recipient per normal HSC sample; 2 recipients per low risk MDS sample), as represented by percentage of human CD45+ chimerism per 500 human HSCs transplanted. (N.S. no significance) (B) Lymphoid (CD19+) and myeloid (CD13+/CD33+) engraftment as a percentage of hCD45+ cells in NSG newborn mice at 16 weeks post-transplant. (*P<0.03; N.S. no significance) (C) Frequency of GMPs out of total human CD34+CD38+ cells engrafted in NSG newborn mice at 16 weeks post-transplant. (**P<0.0007) Error bars represent one standard deviation. "MDS" label represents low risk MDS samples.

Figure 4. Increased apoptosis in low risk MDS GMPs but not in MDS HSCs

(A) Frequency of Annexin V-positive cells in normal (n = 11) and low risk MDS (n = 14) HSCs. (N.S. no significance) (B) Frequency of Annexin V-positive cells in normal (n = 11) and low risk MDS (n = 14) CD34+CD38+ myeloid progenitor cells. (*P<0.03) (C) Frequency of Annexin V-positive cells in normal (n = 11) and low risk MDS (n = 14) GMPs. (**P<0.02) (D) Frequency of Annexin V-positive cells in normal (n = 11) and low risk MDS (n = 14) CMPs. (***P<0.02) (E) Frequency of Annexin V-positive cells in normal (n = 11) and low risk MDS (n = 14) MEPs. (****P<0.02) Error bars represent one standard deviation. "MDS" label represents low risk MDS samples.

Figure 5. Cell-surface CRT is increased on and necessary for the phagocytosis CD34+CD38+ myeloid progenitors in low risk MDS

(A) Cell-surface CRT expression, as expressed by mean fluorescence intensity (MFI) relative to fluorescence-minus-one (FMO) control, on normal (n = 11) and low risk MDS (n = 14) HSCs. (N.S. no significance) (B) Cell-surface CRT expression, as expressed by MFI relative to FMO control, on normal (n = 11) and low risk MDS (n = 14) CD34+CD38+ myeloid progenitor cells and representative histogram. (*P<0.02) (C) Cell-surface CRT expression, as expressed by MFI relative to FMO control, on normal (n = 11) and low risk MDS (n = 14) GMPs. (**P<0.05) (D) Phagocytosis of normal and low risk MDS CD34+CD38+ myeloid progenitors (phagocytic index = # of engulfed green cells / # of macrophages * 100) and representative photomicrographs. (***P<0.02) (E) Phagocytosis of normal and low risk

MDS CD34+CD38+ myeloid progenitors treated with CRT-blocking peptide. (****P<0.003) Error bars represent one standard deviation. “MDS” label represents low risk MDS samples.

Figure 6. CD47 is up-regulated on and necessary for the evasion of phagocytosis by CD34+CD38+ myeloid progenitors in high risk RAEB stage of MDS

(A) CD47 expression, as expressed by MFI relative to FMO control, on normal (n = 9) and RAEB (n = 7) CD34+CD38+ myeloid progenitor cells and representative histogram. (*P<0.03) (B) CD47 expression, as expressed by MFI relative to FMO control, on normal (n = 9) CD34+CD38+ myeloid progenitor cells (3438 bulk), normal MEP, normal GMP, normal CMP, and RAEB (n = 7) CD34+CD38 blasts and RAEB CD34+CD38+ non-blasts. (**P<0.03; N.S. no significance) (C) Cell surface CRT expression, as expressed by MFI relative to FMO control, on normal (n = 9) and RAEB (n = 7) CD34+CD38+ myeloid progenitor cells. (***P<0.04) (D) Cell surface CRT expression, as expressed by MFI relative to FMO control, on normal CD34+CD38+ myeloid progenitor cells (n = 9) and RAEB CD34+CD38+ blasts (n = 7) and RAEB CD34+CD38+ non-blasts (n = 7). (****P<0.04; N.S. no significance) (E) Phagocytosis of normal and RAEB CD34+CD38+ myeloid progenitor cells treated with PBS control, CRT-blocking peptide, or anti-CD47 antibody (B6H12). (#P<0.03, ##P<0.006) Error bars represent one standard deviation.

Supplemental Figure 1.

(A) Frequency of CMPs out of total myeloid progenitors (CMPs + GMPs + MEPs) in normal (n = 34) and low risk MDS (n = 46). (*P<0.02) (B) Frequency of MEPs out of total myeloid progenitors (CMPs + GMPs + MEPs) in normal (n = 34) and low risk MDS (n = 46). (**P<0.0003)(C) Frequency of CMPs out of total lineage negative bone marrow mononuclear cells in normal and low risk MDS bone marrow samples. (N.S. no significance) (D) Frequency of MEPs out of total lineage negative bone marrow mononuclear cells in normal and low risk MDS bone marrow samples. (N.S. no significance) Error bars represent one standard deviation. “MDS” label represents low risk MDS samples.

Supplemental Figure 2.

(A) Cell-surface CRT expression, as expressed by MFI relative to FMO control, on normal (n = 11) and low risk MDS (n = 14) CMPs. (N.S. no significance) (B) Cell-surface CRT expression, as expressed by MFI relative to FMO control, on normal and low risk MDS CMPs. (N.S. no significance) Error bars represent one standard deviation. “MDS” label represents low risk MDS samples.

Supplemental Figure 3.

(A) Phagocytosis of normal and low risk MDS CD34- cells. (N.S. no significance) (B) Phagocytosis of normal and low risk MDS CD34- cells treated with CRT-blocking peptide. (N.S. no significance) Error bars represent one standard deviation. “MDS” label represents low risk MDS samples.

Supplemental Figure 4.

(A) Frequency of GMPs in normal, low risk MDS, and RAEB CD34+CD38+ populations. (*$P<10^{-13}$; **$P<0.002$) (B) CD47 expression, as expressed by MFI relative to FMO control, on normal and low risk MDS CD34+CD38+ myeloid progenitor populations. (N.S. no significance) (C) CD47 expression, as expressed by MFI relative to FMO control, on normal and low risk MDS CMP, GMP, and MEP populations. (N.S. no significance) Error bars represent one standard deviation. “MDS” label represents low risk MDS samples.

Materials and Methods

Human Samples.

Human bone marrow samples were collected from the Stanford Department of Pathology, according to an IRB-approved protocol. Samples were selected from submitted cases solely based on the ability of investigators to obtain and freeze the samples within 72 hours post-collection. The patient samples represented the full spectrum of WHO subtypes of low risk MDS without excess blasts (n=46; includes refractory anemia, refractory anemia with ringed sideroblasts, refractory cytopenia with multilineage dysplasia, and myelodysplastic syndrome not otherwise specified but with fewer than 5% blasts) and high risk MDS (n=7; refractory anemia with excess blasts, includes both WHO RAEB-1 and RAEB-2 subtypes). Controls included a group of non-MDS related cytopenias (n=32) as well as young and elderly normal controls (n=34). The group of MDS patients represented both primary diagnostic samples as well as those with previously known diagnoses. Samples were either processed and frozen as mononuclear cell fractions for future analysis or evaluated immediately. Mononuclear cells were isolated using Ficoll-Paque Plus (GE Healthcare, Fairfield, CT). Mononuclear cells were cryopreserved in 90% FBS/10% DMSO. Cryopreserved samples were thawed and washed with IMDM containing 10% FBS and DNAse I.

Flow Cytometry and Cell Sorting

Antibodies used to stain mononuclear cell suspensions for analysis and sorting of hematopoietic stem and progenitor subpopulations included: PE-Cy5-conjugated anti-human lineage markers (CD3, S4.1; CD4, S3.5; CD7, CD7-6B7; CD8, 3B5; CD10, 5-1B4; CD14, TUK4; CD19, Sj25-C1; CD20, 13.6E12 (Caltag/Invitrogen, Carlsbad, CA); CD2, RPA-2.10; CD11b, ICRF44; CD56, B159; GPA, GA-R2 (BD Biosciences, San Jose, CA)); V450-conjugated anti-CD45RA, MEM56; PE-Cy7-conjugated anti-CD38, HIT2, APC-conjugated anti-CD34, 8G12, PE-conjugated anti-CD123, FITC-conjugated anti-CD90, 5E10, FITC-conjugated or PE-conjugated anti-CD47, B6H12.2 (BD Biosciences), and FITC-conjugated anti-CRT, clone FMC 75. Cells were analyzed and sorted using a FACSAriaII cytometer (BD Biosciences). FlowJo Software (Treestar, Ashland, OR) was used to analyze flow cytometry data.

Mouse Transplantation

NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice obtained originally from The Jackson Laboratory (Bar Harbor, ME) were bred in a Specific Pathogen-Free environment according to a protocol approved by the Stanford Administrative Panel on Laboratory Animal Care. P0-P3 newborn pups were pre-conditioned with 100 rads of gamma irradiation 4-24 hours prior to transplantation(8, 40, 43). Cells resuspended in PBS containing 2% FCS were transplanted intravenously via the anterior facial vein using a 30 or 31 gauge needle.

Analysis of Human Chimerism

The following panel of antibodies (Caltag/Invitrogen, Carlsbad, CA, and BD Biosciences, San Jose, CA) was used for analysis of human engraftment/chimerism: PB-conjugated CD45, HI30; APC-conjugated anti-CD34, 8G12; Alexa Fluor 750-conjugated CD3, S4.1; Alexa Fluor 700-conjugated CD19, SJ25-C1; PE-conjugated CD13, TK1; PE-conjugated CD33, P67.6; PE-Cy5-conjugated GPA, GA-R2; FITC-conjugated CD41a, HIP8. The following panel of antibodies (eBiosciences, San Diego, CA) was used to identify mouse leukocytes and red blood cells, respectively: Alexa488- or PE-Cy7-conjugated CD45.1, A20.1.7; PE-Cy5- or PE-Cy7-conjugated Ter119. Single-cell suspensions were prepared using standard methods from bone marrow of transplanted mice. Red blood cells were lysed using hypotonic solution ACK. For analyses and sorting, except otherwise noted below, cells were stained with the appropriate antibody combinations for 30-60 minutes on ice, and dead cells were excluded by propidium iodide staining.

FISH

Cells were sorted by FACS directly onto a slide for cytogenetic analysis by interphase fluorescence in situ hybridization (FISH). Briefly, slides were fixed in methanol/acetic acid 3:1 for 10-15 minutes, dehydrated in 70%, 80%, 90%, and 100% ethanol sequentially, and air dried. Slides were fixed in 1% neutral buffered formalin for 5 minutes, twice treated with microwave heated sodium citrate buffer, pH 6.0, for 5 minutes, digested with proteinase K (Sigma, St. Louis, MO) for 5 minutes, fixed again in neutral buffered formalin for 5 minutes, dehydrated in 70%, 80%, 90%, and 100% ethanol sequentially, and air dried. The Vysis LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen Probe (Abbott Molecular, Abbott Park, IL) was used for hybridization, according to manufacturer's instructions. After hybridizations, slides were washed in 1x SSC for 5 minutes at 72°C and then 0.05% TBS-Tween for 3 minutes at room temperature, counterstained with DAPI (Sigma, St. Louis, MO).

Nuclei of normal cells contain 4 distinct signals (2 orange and 2 green). Nuclei from MDS patient bone marrow cells with monosomy 7 contain 2 distinct signals (1 orange and 1 green).

Macrophage Isolation and Culture

Leukocyte-enriched human peripheral blood was obtained from the Stanford Blood Center. Mononuclear cells were isolated using Ficoll-Paque (GE HLS) and plated in IMDM+10% human serum (R&D) with PenStrep and Glutamax for 6-day incubation. Peripheral blood derived macrophages are an adherent population following 7-day incubation.

In Vitro Phagocytosis Assay

Human peripheral blood-derived macrophages were harvested by incubation in 10% EDTA (GIBCO) for 30 minutes at 4 degrees and gentle scraping. Macrophages were plated at 5×10^4 cells per well in a 24-well tissue culture plate (Falcon) and incubated for 24 hours. Fresh, serum-free media were added 2 hours prior to introduction of target cells. To prepare target cells, FACS-sorted normal bone marrow and MDS bone marrow CD34+CD38+ and CD34- cells were incubated with 2.5μM CFSE for 10 minutes. Following incubation, target cells were incubated on ice for 15 minutes with either PBS, 2ug/mL CRT-blocking peptide (MBL International), or 10ug/mL anti-CD47 antibody (clone B6H12, eBiosciences). 1×10^5 target cells were added to macrophage-containing wells and incubated for 2 hours. After co-incubation, wells were washed thoroughly with PBS five times and examined under a Leica microscope using an enhanced green fluorescent protein (GFP) filter set. Phagocytic index was calculated using the following formula: phagocytic index = number of ingested cells/number of macrophages*100. At least 300 macrophages were counted per well.

Statistical Analysis.

Statistical analysis using Student's t-test and calculations of specificity and sensitivity were done using Microsoft Excel and/or GraphPad Prism (San Diego, CA) software.

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Author contributions

W.W.P., J.V.P., S.L.S., C.Y.P., and I.L.W. wrote the manuscript. W.W.P., C.Y.P., and I.L.W. designed the experiments. W.W.P. and J.V.P. performed the experiments and analyzed the data. E.A.P., K.S., D.A.A., P.L.G., S.L.S, and C.Y.P. provided clinical samples.

Competing interests

W.W.P., C.Y.P., and I.L.W. filed U.S. Patent Application Serial No. 13/508,319 entitled “Cell Surface Marker Expression in Hematopoietic Stem Cells and Progenitors for the Diagnosis, Prognosis, and Treatment of Myelodysplastic Syndromes.” I.L.W. filed U.S. Patent Application Serial No. 12/321,215 entitled “Methods for Manipulating Phagocytosis Mediated by CD47.” I.L.W. filed a patent application regarding therapeutic and diagnostic methods for manipulating phagocytosis through calreticulin and low density lipoprotein-related receptors. The other authors declare that they have no competing interests.

References

1. Brunning R*, et al.* (2008) Myelodysplastic syndromes/neoplasms. *WHO Classification of Tumours and Haematopoietic and Lymphoid Tissues*, eds Swerdlow S, Campo E, Harris N, Jaffe E, Pileri S, Stein H, Thiele J, & Vardiman J (IARC, Lyon), Vol 2.
2. Hofmann WK & Koeffler HP (2005) Myelodysplastic syndrome. *Annu Rev Med* 56:1-16.
3. Greenberg P*, et al.* (1997) International scoring system for evaluating prognosis in myelodysplastic syndromes. (Translated from eng) *Blood* 89(6):2079-2088 (in eng).
4. Greenberg PL*, et al.* (2012) Revised international prognostic scoring system for myelodysplastic syndromes. (Translated from eng) *Blood* 120(12):2454-2465 (in eng).
5. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, & Peault B (1992) Isolation of a candidate human hematopoietic stem-cell population. (Translated from eng) *Proc Natl Acad Sci U S A* 89(7):2804-2808 (in eng).
6. Uchida N*, et al.* (1998) HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. (Translated from eng) *Proc Natl Acad Sci U S A* 95(20):11939-11944 (in eng).
7. Manz MG, Miyamoto T, Akashi K, & Weissman IL (2002) Prospective isolation of human clonogenic common myeloid progenitors. *Proc Natl Acad Sci U S A* 99(18):11872-11877.
8. Majeti R, Park CY, & Weissman IL (2007) Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. (Translated from eng) *Cell Stem Cell* 1(6):635-645 (in eng).
9. Nilsson L*, et al.* (2002) Involvement and functional impairment of the CD34(+)CD38(-)Thy-1(+) hematopoietic stem cell pool in myelodysplastic syndromes with trisomy 8. *Blood* 100(1):259-267.
10. Nilsson L*, et al.* (2007) The molecular signature of MDS stem cells supports a stem-cell origin of 5q myelodysplastic syndromes. *Blood* 110(8):3005-3014.
11. Tehranchi R*, et al.* (2010) Persistent Malignant Stem Cells in del(5q) Myelodysplasia in Remission. (Translated from Eng) *N Engl J Med* 363(11):1025-1037 (in Eng).
12. Will B*, et al.* (2012) Stem and progenitor cells in myelodysplastic syndromes show aberrant stage-specific expansion and harbor genetic and epigenetic alterations. (Translated from eng) *Blood* 120(10):2076-2086 (in eng).
13. Raza A*, et al.* (1997) Cell cycle kinetic studies in 68 patients with myelodysplastic syndromes following intravenous iodo- and/or bromodeoxyuridine. (Translated from eng) *Exp Hematol* 25(6):530-535 (in eng).
14. Mundle SD*, et al.* (1996) Indication of an involvement of interleukin-1 beta converting enzyme-like protease in intramedullary apoptotic cell death in the bone marrow of patients with myelodysplastic syndromes. (Translated from eng) *Blood* 88(7):2640-2647 (in eng).
15. Shetty V*, et al.* (1996) Measurement of apoptosis, proliferation and three cytokines in 46 patients with myelodysplastic syndromes. (Translated from eng) *Leukemia research* 20(11-12):891-900 (in eng).
16. Rajapaksa R, Ginzton N, Rott LS, & Greenberg PL (1996) Altered oncoprotein expression and apoptosis in myelodysplastic syndrome marrow cells. (Translated from eng) *Blood* 88(11):4275-4287 (in eng).
17. van de Loosdrecht AA*, et al.* (2009) Standardization of flow cytometry in myelodysplastic syndromes: report from the first European LeukemiaNet working

conference on flow cytometry in myelodysplastic syndromes. (Translated from eng) *Haematologica* 94(8):1124-1134 (in eng).
18. Chao MP*, et al.* (2010) Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. (Translated from Eng) *Sci Transl Med* 2(63):63ra94 (in Eng).
19. Gardai SJ*, et al.* (2005) Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. (Translated from eng) *Cell* 123(2):321-334 (in eng).
20. Orr AW*, et al.* (2003) Low density lipoprotein receptor-related protein is a calreticulin coreceptor that signals focal adhesion disassembly. (Translated from eng) *J Cell Biol* 161(6):1179-1189 (in eng).
21. Jaiswal S*, et al.* (2009) CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. (Translated from eng) *Cell* 138(2):271-285 (in eng).
22. Chan KS*, et al.* (2009) Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells. (Translated from eng) *Proc Natl Acad Sci U S A* 106(33):14016-14021 (in eng).
23. Chao MP*, et al.* (2010) Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. (Translated from eng) *Cell* 142(5):699-713 (in eng).
24. Chao MP*, et al.* (2011) Therapeutic antibody targeting of CD47 eliminates human acute lymphoblastic leukemia. (Translated from eng) *Cancer Res* 71(4):1374-1384 (in eng).
25. Miyamoto T, Weissman IL, & Akashi K (2000) AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. (Translated from eng) *Proc Natl Acad Sci U S A* 97(13):7521-7526 (in eng).
26. Reya T, Morrison SJ, Clarke MF, & Weissman IL (2001) Stem cells, cancer, and cancer stem cells. (Translated from eng) *Nature* 414(6859):105-111 (in eng).
27. Weissman I (2005) Stem cell research: paths to cancer therapies and regenerative medicine. *JAMA* 294(11):1359-1366.
28. Chao MP, Majeti R, & Weissman IL (2011) Programmed cell removal: a new obstacle in the road to developing cancer. (Translated from eng) *Nat Rev Cancer* 12(1):58-67 (in eng).
29. Jamieson CH*, et al.* (2004) Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. (Translated from eng) *N Engl J Med* 351(7):657-667 (in eng).
30. Jamieson CH*, et al.* (2006) The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. (Translated from eng) *Proc Natl Acad Sci U S A* 103(16):6224-6229 (in eng).
31. Rossi DJ, Jamieson CH, & Weissman IL (2008) Stems cells and the pathways to aging and cancer. (Translated from eng) *Cell* 132(4):681-696 (in eng).
32. Abrahamsson AE*, et al.* (2009) Glycogen synthase kinase 3beta missplicing contributes to leukemia stem cell generation. (Translated from eng) *Proc Natl Acad Sci U S A* 106(10):3925-3929 (in eng).
33. Jan M*, et al.* (2012) Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. (Translated from eng) *Sci Transl Med* 4(149):149ra118 (in eng).

34. Bhatia M, Wang JC, Kapp U, Bonnet D, & Dick JE (1997) Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. (Translated from eng) *Proc Natl Acad Sci U S A* 94(10):5320-5325 (in eng).
35. Peault B, Weissman IL, Buckle AM, Tsukamoto A, & Baum C (1993) Thy-1-expressing CD34+ human cells express multiple hematopoietic potentialities in vitro and in SCID-hu mice. (Translated from eng) *Nouv Rev Fr Hematol* 35(1):91-93 (in eng).
36. McCune JM*, et al.* (1988) The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. (Translated from eng) *Science* 241(4873):1632-1639 (in eng).
37. Negrin RS*, et al.* (2000) Transplantation of highly purified CD34+Thy-1+ hematopoietic stem cells in patients with metastatic breast cancer. (Translated from eng) *Biol Blood Marrow Transplant* 6(3):262-271 (in eng).
38. Murray L*, et al.* (1994) Analysis of human hematopoietic stem cell populations. (Translated from eng) *Blood Cells* 20(2-3):364-369; discussion 369-370 (in eng).
39. Pang WW*, et al.* (2011) Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. (Translated from eng) *Proc Natl Acad Sci U S A* 108(50):20012-20017 (in eng).
40. Park CY, Majeti R, & Weissman IL (2008) In vivo evaluation of human hematopoiesis through xenotransplantation of purified hematopoietic stem cells from umbilical cord blood. (Translated from eng) *Nature protocols* 3(12):1932-1940 (in eng).
41. Sternberg A*, et al.* (2005) Evidence for reduced B-cell progenitors in early (low-risk) myelodysplastic syndrome. (Translated from eng) *Blood* 106(9):2982-2991 (in eng).
42. Lagasse E & Weissman IL (1994) bcl-2 inhibits apoptosis of neutrophils but not their engulfment by macrophages. (Translated from eng) *J Exp Med* 179(3):1047-1052 (in eng).
43. Ishikawa F*, et al.* (2005) Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. *Blood* 106(5):1565-1573.